Piscine orthoreovirus: Distribution, characterization and experimental infections in salmonids

Vendramin, Niccolò

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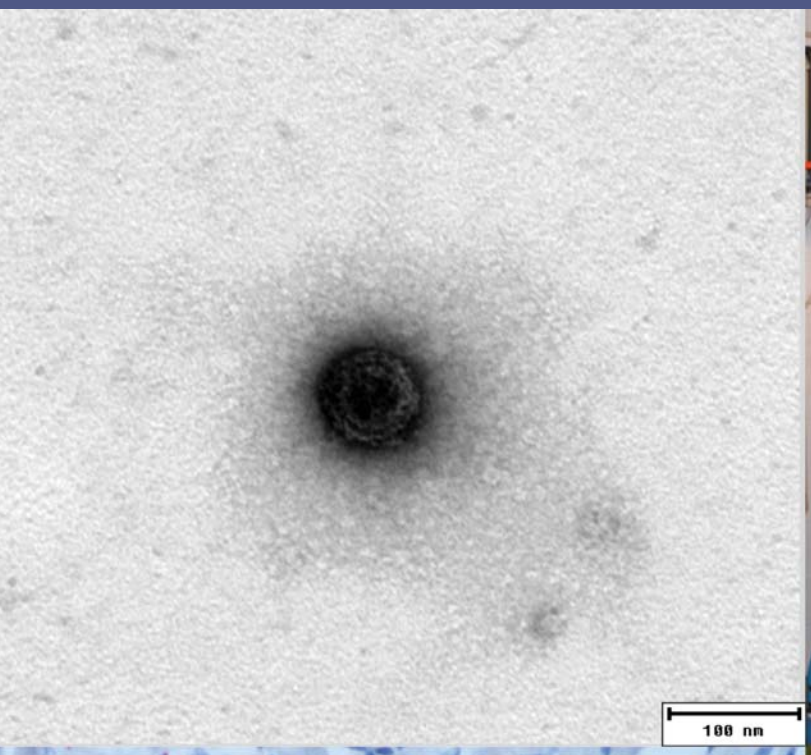
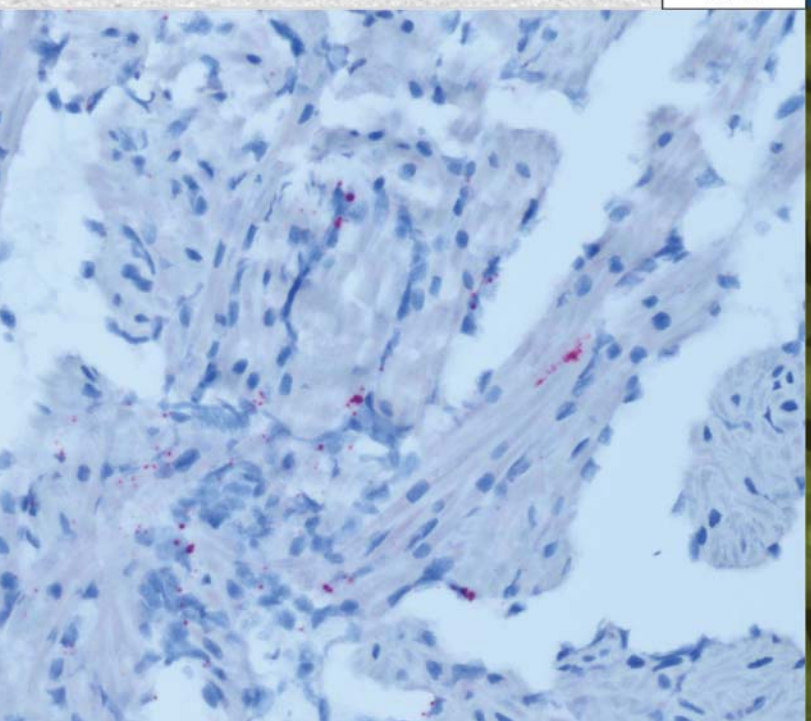
Piscine orthoreovirus

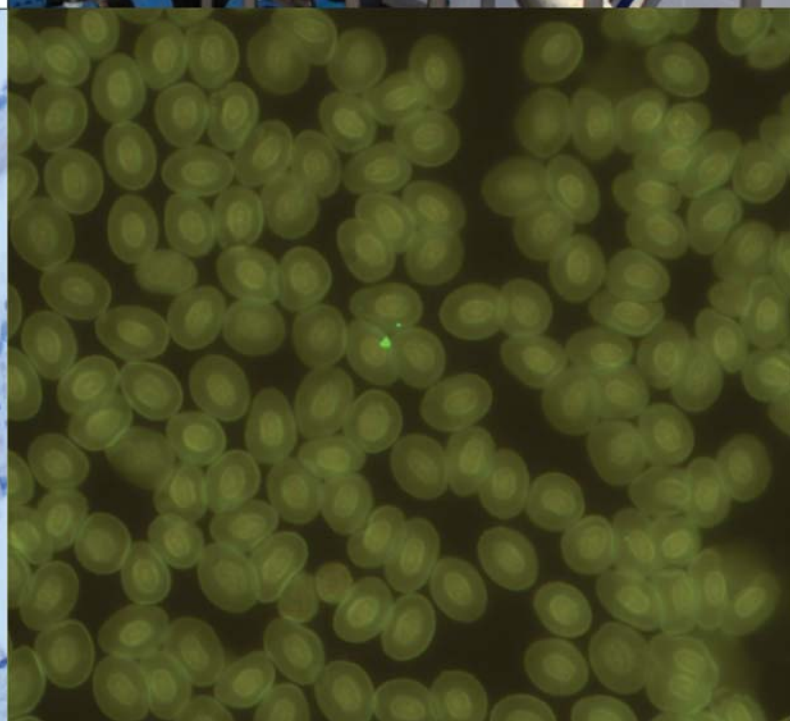
Distribution, characterization and experimental infections in salmonids

By Niccolò Vendramin

PhD Thesis







Piscine orthoreovirus. Distribution, characterization and experimental infections in salmonids

Philosophiae Doctor (PhD) Thesis

Niccolò Vendramin

Unit for Fish and Shellfish diseases

National Institute for Aquatic Resources

DTU-Technical University of Denmark

Kgs. Lyngby 2018

You can't always get what you want
But if you try sometime you might find
You get what you need

1969 The Rolling Stones

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Acknowledgements

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Thanks to my parents, my sisters and all my friends who have cheered along the way, and made this project a reality!

Copenhagen, November 2018

Niccoló Vendramin

List of abbreviations

ARV	Avian orthoreovirus
BKD	Bacterial kidney disease
CD8	Cluster of differentiation 8
CMS	Cardiomyopathy syndrome
DKK	Danish krona
DNA	Deoxyribonucleic acid
dsRNA	Double stranded RNA
EIBS	Erythrocytic inclusion body syndrome
EM	Electron microscopy
ERM	Enteric red mouth
EU	European union
FCR	Food conversion rate
GCRV	Grass carp reovirus
HSMI	Heart skeletal muscle inflammation
ICTV	International committee on taxonomy of viruses
IFAT	Immunofluorescence antibody test
IFN	Interferon
IHC	Immunohistochemistry
IHN	Infectious haematopoietic necrosis
IPN	Infectious pancreatic necrosis
ISA	Infectious salmon anemia
ISRT	Idyopatic syndrome of Rainbow trout
ISVP	Infectious subviral particles
IUdR	Iododeoxyuridine
JAM-A	Junction adhesion molecule A
LMBRV	Largemouth bass orthoreovirus
MHC	Major histocompatibility complex
MRV	Mammalian orthoreovirus
NGS	Next generation sequencing
PAMP	Pathogen associated molecular pattern
PD	Pancreas disease
PKD	Proliferative kidney disease
PKR	Protein kinase R
PKZ	Protein kinase Z
PMCV	Piscine myocarditis virus
PRV	Piscine orthoreovirus
qPCR	Quantitative polymerase chain reaction
RAS	Recirculating aquaculture system

RBC	Red blood cells
RdRp	RNA dependant RNA polymerase
RMS	Red mark syndrome
RNA	Ribonucleic acid
RTFS	Rainbow trout fry syndrome
SAV	Salmonid alphavirus
ssRNA	Single stranded RNA
T	Triangulation number
TiLV	Tilapia lake virus
TLR	Toll like receptor
VER	Viral encephalopathy and retinopaty
VHS	Viral haemorragic septicaemia
VNN	Viral nervous necrosis
VP	Viral protein

List of papers

Paper 1

Infection experiments with novel *Piscine orthoreovirus* from Rainbow trout (*Oncorhynchus mykiss*) in salmonids

Helena Hauge*, Niccoló Vendramin*, Torunn Taksdal, Anne Berit Olsen, Øystein Wessel, Susie Sommer Mikkelsen, Anna Luiza Farias Alencar, Niels Jørgen Olesen, Maria Krudtaa Dahle

*These authors contributed equally to this work

PLoS ONE 12(7): e0180293 2017

Paper 2

Molecular and Antigenic Characterization of *Piscine orthoreovirus* (PRV) from Rainbow Trout (*Oncorhynchus mykiss*)

Dhamotharan Kannimuthu, Niccoló Vendramin, Turhan Markussen, Øystein Wessel, Argelia Cuenca, Ingvild Bergman Nyman, Anne Berit Olsen, Tortsein Tengs, Maria Krudtaa Dahle, Espen Rimstad.

Viruses 2018, 10(4)

Paper 3

***Piscine orthoreovirus* subtype 3 (PRV-3) causes heart inflammation in Rainbow trout (*Onchorynchus mykiss*)**

Niccoló Vendramin*, Dhamotharan Kannimuthu*, Anne Berit Olsen, Argelia Cuenca, Lena Hammerlund Teige, Øystein Wessel, Tine Moesgaard Iburg, Maria Krudtaa Dahle, Espen Rimstad, Niels Jørgen Olesen

In press at Veterinary Research 2019

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Presence and genetic variability of Piscine orthoreovirus genotype 1 (PRV-1) in wild salmonids in Northern Europe and Atlantic Ocean

Niccoló Vendramin, Argelia Cuenca, Juliane Sørensen, Anna Luiza Farias Alencar, Debes Christiansen, Jan Arge Jacobsen, Charlotte Axen, François Lieffrig , Neil M. Ruane, Patrick Martin, Timothy Sheehan, Tine Moesgaard Iburg, Espen Rimstad, Niels Jørgen Olesen

To be submitted to Journal of fish disease in 2018

Paper 5

***Piscine orthoreovirus* infection in Atlantic salmon (Salmo salar) protects against subsequent challenge with infectious hematopoietic necrosis virus (IHNV).**

Niccoló Vendramin, Anna Luisa Farias Alencar, Tine Moesgaard Iburg, Maria Krudtaa Dahle, Øystein Wessel, Anne Berit Olsen, Espen Rimstad, Niels Jørgen Olesen

Vet Res. 2018; 49(1):30.

SUMMARY

Piscine orthoreovirus (PRV) belongs to the family *Reoviridae* and is most closely related to the genus *Orthoreovirus*.

Piscine orthoreovirus are causative agents of emerging diseases for salmonid aquaculture worldwide.

This viral species currently counts three different subtypes, each one with preferential host. PRV-1 is the causative agent of heart and skeletal muscle inflammation (HSMI) in Atlantic salmon (*Salmo salar*) and is associated with jaundice syndrome in farmed Chinook salmon (*Oncorhynchus tshawytscha*). PRV-2 causes erythrocytic inclusion body syndrome (EIBS) in Coho salmon (*Oncorhynchus kisutch*). PRV-3 causes heart pathology resembling HSMI in Rainbow trout (*Onchorynchus mykiss*). In Europe only PRV-1 and PRV-3 are present

PRV-3 was firstly discovered in 2013 in Norway during disease outbreaks affecting farmed Rainbow trout. The first series of experimental trials were performed to assess its pathogenicity and pathogenesis in Rainbow trout and Atlantic salmon.
The Norwegian PRV-3 isolate has been further characterized analyzing its genome and antigenic features. An experimental infection study with purified virus demonstrated that PRV-3 infection in Rainbow trout induces pathological heart lesions similar to HSMI, and thus fulfil causative relationship. Furthermore, the infection upregulates IFN production and induces a specific antibody response in later phases. In late 2017 the presence of PRV-3 was also reported in different countries in Europe including Scotland, Germany, France, Italy and Denmark. Interestingly, these viral isolates appear to be genetically distinct from the Norwegian isolate leading to the proposition of two separate clades within PRV-3 viral type (PRV-3a and PRV-3b).

PRV-1 is prevalent in farmed Atlantic salmon in sea water in Europe. The prevalence of the virus has been investigated in wild salmon stocks to expand the knowledge of its epidemiology. PRV can induce a systemic antiviral immune response, which may affect the outcome of a secondary virus infection. Infectious haematopoietic necrosis virus (IHNV) causes major challenges to the Atlantic salmon industry in Canada, requiring the implementation of DNA vaccination to control the disease. IHNV is not present in Atlantic salmon farmed in Europe and feared as a major risk for the aquaculture industry, and therefore we have assessed the potential interaction of these two pathogens in Atlantic salmon in the co-challenge trial.

SAMMENDRAG (Summary in Danish)

Piscine orthoreovirus (PRV) tilhører til familien *Reoviridae* og er nært beslægtet med genus *Orthoreovirus*.

Piscine orthoreovirus er årsag til alvorlig sygdom hos laksefisk i akvakultur på verdensplan.

Dette virus omfatter tre forskellige undertyper, med hver sin foretrukne vært. PRV-1 er den tilgrundliggende faktor for heart and skeletal muscle inflammation (HSMI) i laks (*Salmo salar*) og er forbundet med Jaundice syndrome hos opdrættede Chinook-laks (*Oncorhynchus tshawytscha*). PRV-2 forårsager Erythrocytic inclusion body syndrome (EIBS) i Coho laks (*Oncorhynchus kisutch*) i Japan. PRV-3 forårsager hjertepatologi, der ligner HSMI i regnbueørred (*Onchorynchus mykiss*). I Europa forefindes kun PRV-1 og PRV-3.

PRV-3 blev først påvist i 2013 i Norge under et sygdomsudbrud, der angrebe opdrættede regnbueørred. Den første serie af experimentelle forsøg blev udført for at vurdere dets patogenicitet og patogenese i regnbueørred og laks. Det norske PRV-3 isolat er yderligere karakteriseret ved at analysere af genomet og antigene egenskaber. Et eksperimentel infektionsstudie i regnebueørred med oprenset virus viste, at PRV-3-infektion i forårsagede patologiske hjerte læsioner svarende til HSMI og understøtter dermed årsagssammenhæng. Desuden øger infektionen IFN-produktion og forårsager specifikt antistofrespons i senere faser. I slutningen af 2017 blev tilstedeværelsen af PRV-3 også rapporteret i forskellige lande i Europa, herunder Skotland, Tyskland, Frankrig, Italien og Danmark. Interessant nok synes disse vira at være genetisk forskellige fra det norske isolat, hvilket har medført at forslag om to separate clades inden for PRV-3 viraltypen (PRV-3a og PRV-3b).

I Europa er PRV-1 dominerende i opdrættet Atlanterlaks i havvand. Udbredelse af PRV-1 er blevet undersøgt i vilde laksebestande for at øge kendskabet til epidemiologien. PRV kan fremkalde et systemisk antiviralt immunrespons, hvilket kan påvirke resultatet af en sekundær virusinfektion. Infectious haematopoietic necrosis virus (IHNV) forårsager store udfordringer for laksindustrien i Canada, der kræver implementering af DNA-vaccination til bekæmpelse af sygdommen. IHNV er ikke til stede i Atlanterhavslaks opdrættet i Europa og frygtes som en stor risiko for akvakultursektoren, og derfor har vi vurderet den potentielle interaktion mellem disse to patogener i Atlanterhavslaks i et experimental forsøg.

INTRODUCTION

Aquaculture production in Europe and Denmark

The global demand for animal protein increases with increasing human population, estimated to reach 8 billion in 2023 (World Population Prospects 2017). Aquaculture is among the fastest growing food-producing sector [1] together with poultry production. The key aspect of this trend the efficient food conversion ratio (FCR) of these two groups of farmed animals (Fig. 1) [2]. Currently, aquaculture production accounts for nearly fifty percent of the world's food fish supply.

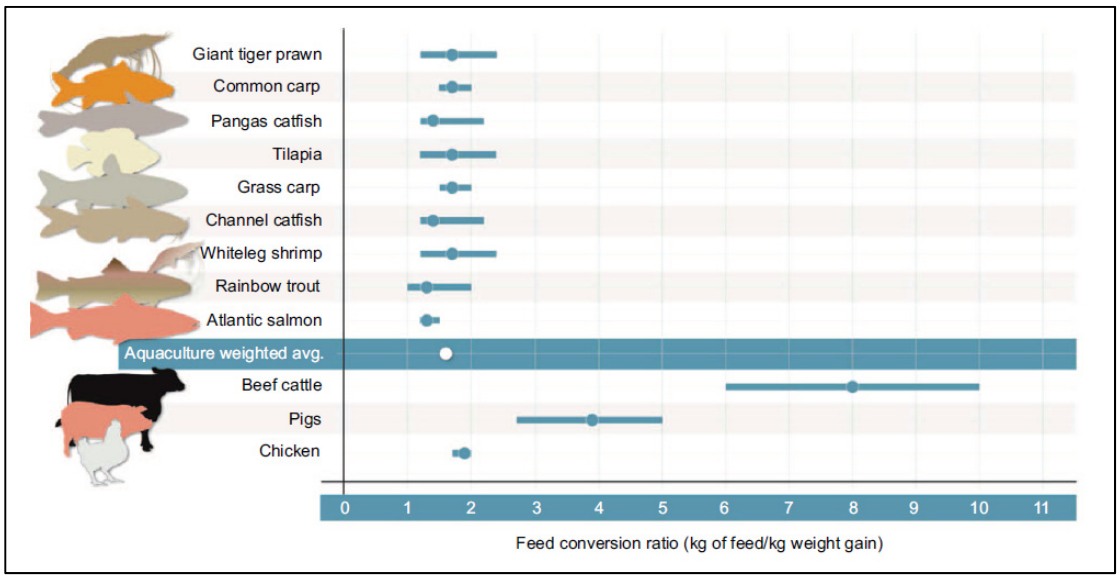

Figure 1. Feed conversion ratios for selected aquatic and terrestrial farmed animal species. Dots represent means and bars indicate range. Lower values signify higher efficiency. From Fry et al., 2018 [2]

In Europe, aquaculture production is dominated by salmonids represented by Atlantic salmon (*Salmo salar* L.) and Rainbow trout (*Onchorynchus mykiss* Walbaum). The total European production of fish by aquaculture is estimated to be 2,327,082 tons in 2016. Together with the two salmonids aforementioned, Gilthead seabream (*Sparus aurata* L.), European seabass (*Dicentrarchus labrax* L.) and common carp (*Cyprinus carpio* L.), represented 95% of the total European production in 2016 [3]. Within EU, two major production systems at the national level can be distinguished; in one hand countries where the industry produce large quantity of fish for food consumption, such as Norway, Turkey, Scotland, Ireland, and Faroe Islands [3]; in other countries the industry has developed aquaculture systems for the production of high-quality live fish (eyed eggs) for global export. Denmark, for example, annually produce approximately 276 million Rainbow trout eggs [4, 5] worth 18.5 million DKK [6]. Two third of this production is exported, thanks to the certified high health standard; the major buyer of Danish eyed-eggs is currently Chile, which yearly imports approximately 70 million eggs [7]. The strategic national aim is to increase the Atlantic salmon production in Norway fivefold by 2050, while aquaculture production in Denmark aims to reach 100.000 tons by 2025. These ambitious goals require control of both biological and technical challenges.

Infectious diseases in aquaculture

Despite the continuous development and increase of aquaculture production on the global scale [1], production appear has stagnated in Europe since 2014 [3]. In modern aquaculture large number of fish (up to 250.000 specimens in a single cage) are confined in the space of farming unit (tank, pond net pen). The number of interactions between hosts in high confinement is drastically higher from the natural environment. This condition puts pressure on the infectious agent, allowing its evolution towards higher pathogenicity [8]. A significant contributor to this stagnation is the impact and treatment of infectious diseases. Historically, infectious diseases have significantly contributed to the development of aquaculture, and only when preventive measures and control strategies have been effectively implemented the production has become sustainable.

Examples of measures and control strategies are:

1- the implementation of vaccination against the bacterial diseases as classical and cold water vibriosis and furunculosis in farmed salmonids Norway that has minimized related disease outbreaks [9].

2- The implementation of regulation such as zoning, biosecurity measures at farm level and intensive surveillance after massive infectious salmon anemia (ISA) outbreak in Faroe Island in 2006 (D. Christiansen, personal communication) (Fig. 2).

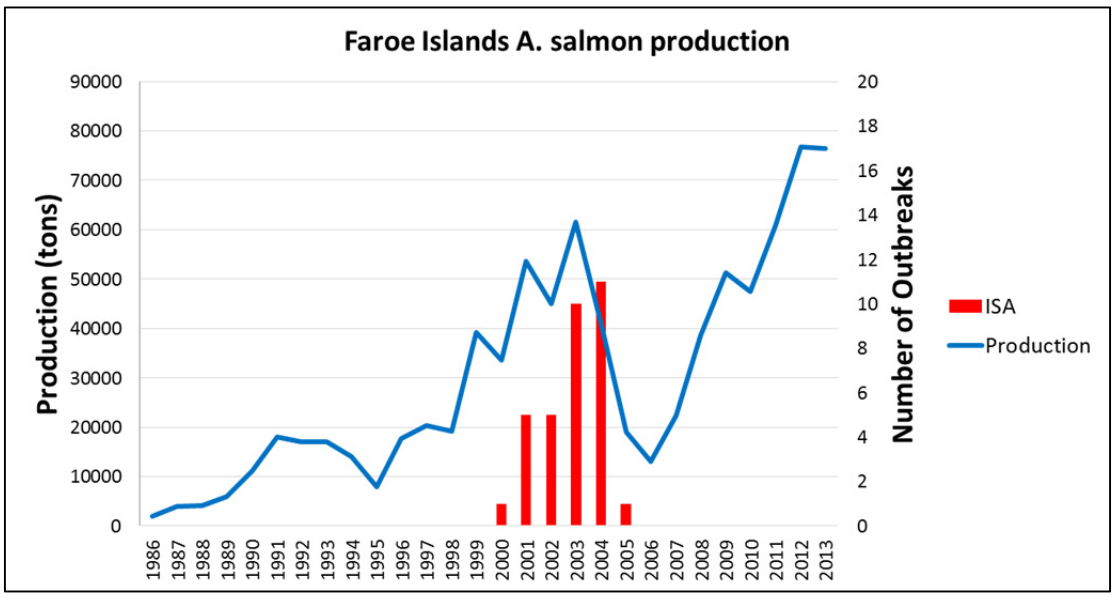

Figure 2. Production of Atlantic salmon in Faroe Islands control from 1986 to 2013 and number if ISA outbreaks. Graph kindly provided by Debes Christiansen FVO.

3- The implementation of regulation such as zoning, biosecurity measures at farm level and intensive surveillance after ISA outbreak in Chile starting in 2007 when production diminished by roughly 60% in 2 years from 388.000 tons in 2008 to 120.000 tons in 2010 (Fig 3)

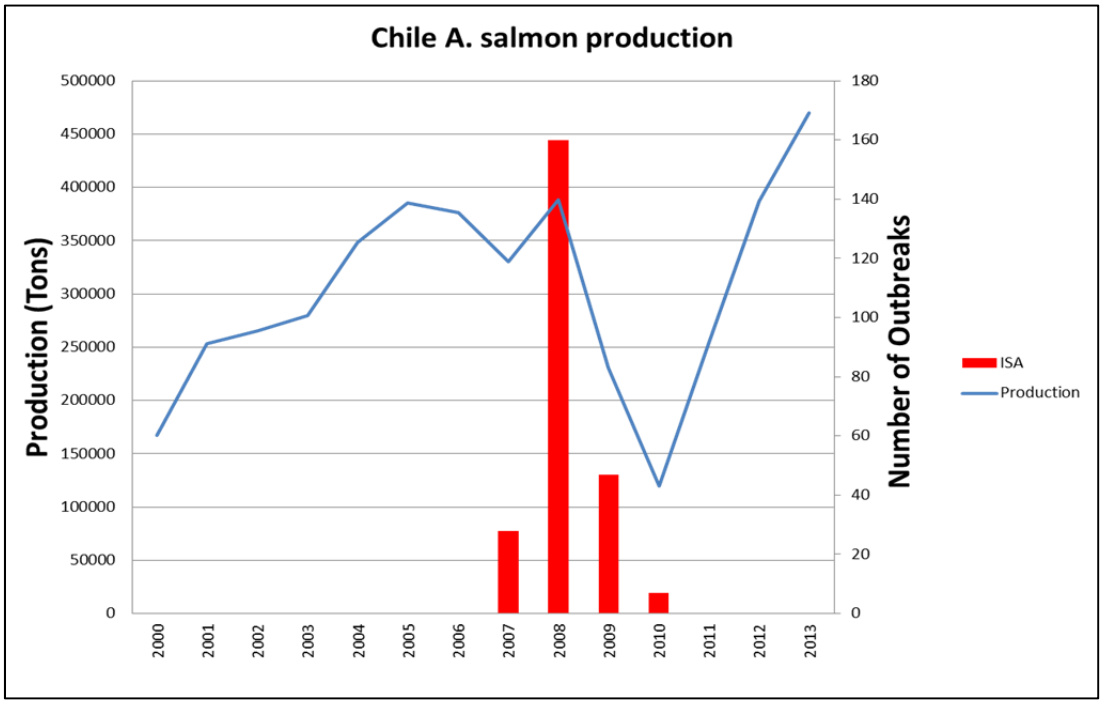

Figure 3. Production of Atlantic salmon in Chile in relation to ISA prevention and control from 2000 to 2013. (production data from FIGIS; ISA outbreaks from Godoy et al., 2013 [10]

Infectious diseases affecting Rainbow trout production in Denmark

The production of Rainbow trout in Denmark is approximately 35.000 tons, where 21.000 tons are portion sized Rainbow trout (fish of approx. 300 grams) produced in freshwater and 14.000 tons of large Rainbow trout (fish of approx. 4 kg) produced seasonally in cages in sea- or brackish- water.

In Denmark there are currently 166 freshwater farms; 131 of these have traditional flow-through water supply where water comes from rivers or boreholes and flow through earth ponds or raceways where fish are cultivated. The outflow is often discarded directly into the river after filtration and removal of the organic material; the remaining 35 farms use recirculating aquaculture systems (RAS).

In RAS the water is recirculated to different degrees (from partly to fully). In order to enable efficient fish farming in RAS, water treatment is required. Each treatment step targets a limiting waste component, in a pre-defined order, first particles and organic matters are removed, next ammonia, nitrates and nitrates are removed by the biofilter. The removal of each limiting waste component reduces the needs of water intake for the system. Based on the system water exchange rate RAS can be classified as follow: flow through (>50 m^3/kg feed), re-use (1–50 m^3/kg feed), conventional recirculation (0.1–1 m^3/kg feed) and 'next generation' or 'innovative' RAS (<0.1

m^3/kg feed). RAS systems have been developed to respond to the increasing environmental regulations in countries with limited access to land and water [11].

The RAS in Danish farms further divides into type 1 or re-use (18 farms) and type 3 or innovative RAS (17 farms). The production capacity per farm change significantly according to farm type, in 2016 it was calculated that traditional farms in Denmark could produce annually 127 tons, while RAS type 1 and RAS type 3 could produce 1.255 tons and 3.681 tons, respectively [12].

Denmark has invested significant efforts and resources to make high fish health standard a national hallmark and has eradicated important fish diseases at country or farm levels. As a result, Denmark is the only example worldwide of a country where viral haemorragic septicaemia (VHS) was endemic and later was eradicated. Denmark has achieved VHS free status at country level [13, 14]. Furthermore, Denmark is also free of infectious hematopoietic necrosis (IHN) according to European legislation [15, 16]. Finally, there are national voluntarily programs to maintain status free of infectious pancreatic necrosis (IPN) and bacterial kidney disease (BKD) for selected farms.

However, common production diseases still challenge the aquaculture production in Denmark.

Among these, the disease caused by *Flavobacterium psychrophilum,* also known as Rainbow trout fry syndrome (RTFS) or flavobacteriosis, is probably the most important bacterial disease [17–19]. Despite the availability of vaccines, other relevant pathogens remain *Aeromonas salmonicida* causing furunculosis [20] and *Yersinia ruckerii* causing enteric red mouth (ERM) [21].

Parasitic infestation caused by *Ichtiopthirius multifilis* is still considered an important problem in Rainbow trout production in Denmark, and similarly for *Tetracapsula bryosalmonae* causing proliferative kidney disease (PKD), which is limited to few flow-through farms [22, 23]. The emergence of nodular gill disease associated with Amoeba parasite [24] is also challenging Rainbow trout production in Denmark.

There are a number of parasites considered to be of less significance, but nevertheless can be problematic if they are present in high amounts. Examples are *Gyrodactylus* spp. (not *G. salaris*), and various motile ciliates like *Chilodonella* and *Trichodina* and immobile ciliates that stick to the fish like *Glossatella*, *Scyphidia,* and *Trichophyra, Spironucleus*.

Finally, the viral disease IPN is present in Denmark [25].

The overarching goal of obtaining high health standards has led to the eradication of highly pathogenic pathogens and partly or entirely control of production diseases. But, new risks are posed by emerging infections and diseases. The preventive measures can be established at different levels such as farm level, zone level or country level [26, 27].

Emerging diseases in aquaculture

How do we define disease? This question still raises considerable debate worldwide, and especially in human medicine, the definition of disease and health is integrated into a complex setting where human behavior and social science are deeply integrated [28].

In veterinary science, "diseas is defined as an impairment of the normal state of an animal that interrupts or modifies its vital functions [29].

In aquaculture, infectious viral diseases have traditionally been associated with acute outbreaks causing high mortality; examples are VHS in Rainbow trout, infectious salmon anemia (ISA) in Atlantic salmon, viral encephalopathy and retinopathy (VER) in European sea bass, etc. Recently, the recognition of more subtle chronic infections has shifted the focus from "mortality" which after all is a rather crude and rough way of assessing the impacts of a disease, to various clinical observations. These include food conversion rate (FCR), aberrant swimming, inappetence, reduced expected growth rate, interaction with other diseases, reduced tolerance to hypoxia, and in case of salmonids interaction with smoltification process. Finally, it should be remarked that the assessment of fish behavior in aquaculture systems, in general, is rather limited due to water depth, water transparency, etc., so infections leading to histopathological lesions which are not sufficient to cause mortality or significant changes in behavior might simply be missed.

The definition of ''emerging disease'' is three-folded. It is "emerging", if it is a new disease, where "new" indicates a disease previously unknown or undescribed. But, it is also considered as "emerging" a new manifestation of a known disease, due to increased severity or change in the host species. Finally, the appearance of a disease in a new geographical area is also considered as "emerging" [26].

In aquaculture, many risk factors favor the emergence, establishment, and spread of diseases. Among these is the interaction between wild and farmed fish, which increase the possibility of exchanging pathogens; the stocking density of farmed fish which are high compared to wild stocks and favor the transmission of pathogenic pathogens; the trade of live animals which can introduce pathogens into new geographical areas, etc.

The introduction of new species in aquaculture, especially when the fish originally comes from the wild has favored the emergence of new pathogens. This has recently occurred with the emergence of VHS genotype IV for the first time in Europe [30] and of a new Ranavirus that both have been detected in Lumpfish (*Cyclopterus lumpus*) in Iceland (Stagg et al., 2018 submitted). In Norway, a new flavivirus has been associated with pathologic lesions in the liver [31]. There has been a sudden increase in farming of Lumpfish due to its popularity as cleaner fish in salmon farming until now the broodfish have a wild origin. Another example of the emergence of new diseases in aquaculture linked to new species introduced to aquaculture is the emergence of Tilapia Lake virus-(TiLV), which is associated with severe diseases in Tilapia farming and has spread in several countries worldwide [32–35].

Emerging diseases also affect well-established aquaculture practices such as Atlantic salmon farming in northern Europe. In recent years a number of viral pathogens, mostly uncultivable have affected Atlantic salmon aquaculture. Examples are cardiomyopahty syndrome (CMS) associated

with Piscine myocarditis virus (PMCV) [36], Salmon Gill Disease Pox Virus [37, 38] and *Piscine orthoreovirus* (PRV) causing heart and skeletal muscle inflammation (HSMI) [39]. A significant contribution to the detection and characterization of uncultivable pathogens has been provided by next-generation sequencing [40, 41].

In Denmark, two different diseases have recently emerged. Red mark syndrome (RMS) [42], is associated with an intracellular bacteria belonging to the newly described *Midichloriaceae* family [43, 44]. This infection appears to be prevalent in the country, does not cause significant mortality and has benign prognosis; its major impact is impeding a programmed production cycle as affected fish develop self-healing wounds in the skin, which impair marketing of the fish.

Another disease characterized by severe mortality in RAS has recently emerged and is associated with a newly described PRV subtype 3, which is the core topic of this Ph.D. study. The virus was discovered in 2013 in Norway in association with the disease outbreak in Rainbow trout [45]. The virus was investigated in Denmark in 2016 without being detected. In 2017, after its discovery in association with the severe disease outbreak, the virus has spread in the country and in Europe.

Orthoreoviruses

Piscine orthoreovirus belongs to the *Reoviridae* family, genus *Orthoreovirus.*

The taxonomy of the family *Reoviridae* is complex, currently comprising 15 recognized genera within two subfamilies of viruses. The reovirus' genomes are composed of multiple (10-12) segments of double-stranded RNA (dsRNA) surrounded by one, two or three concentric layers of capsid proteins [46]. The arrival of modern sequencing techniques and bioinformatics analysis have revolutionized and rationalized virus taxonomy. Re-organization of the viral taxonomy including the one of reoviruses can be anticipated in the next decade.

Within the *Reoviridae* family, individual virus species infect a remarkable variety of hosts, including mammals, birds, reptiles, amphibians, fish, molluscs, crustaceans, insects, plants, and fungi. The root term "reo-" is an acronym for "respiratory enteric orphan" which was coined because the first members of the family were identified in the respiratory and the enteric tracts of animals and humans as "orphans"—that is, not associated with any disease. The different viral genera within this family can be distinguished on the basis of their capsid structure; the number and size of genome segments; the viral host range and associated diseases; the virus' antigenic properties, and by the nucleotide sequence of their genomes. [46].

Reoviridae family includes two subfamilies, *Sedoreovirinae* and *Spinareovirinae*. The subfamily *Spinareovirinae* includes viruses that display large spikes or turrets situated at the 12 icosahedral vertices of either the virus or core particle. This family contains two viral genera with relevance for finfish, namely *Aquareovirus* and *Orthoreovirus*. These genera has a common ancestor, as supported by sequence analysis of genomic segment 7 [47]. The genus *Aquareovirus* includes seven species, named from A to F. *Aquareovirus* have been isolated from a wide variety of finfish including salmonids, cyprinids and flatfish. Aquareovirus A has been isolated from Chum salmon (*Oncorhynchus keta*) and Atlantic salmon, Aquareovirus C in Golden shiner (*Notemigonus crysoleucas*), Aquareovirus G in Grass carp (*Ctenopharyngodon idella*). Currently, two *Aquareovirus* with unassigned species has been isolated in flatfish as turbot (*Scophtalmus maximus*) and Atlantic Halibut (*Hyppoglossus hyppoglossus*) [48]. Notably, *Aquareovirus* can be cultivated on cell culture, these virus express fusogenic proteins which induce the formation of syncytia in cell culture as typical CPE (Fig. 4).

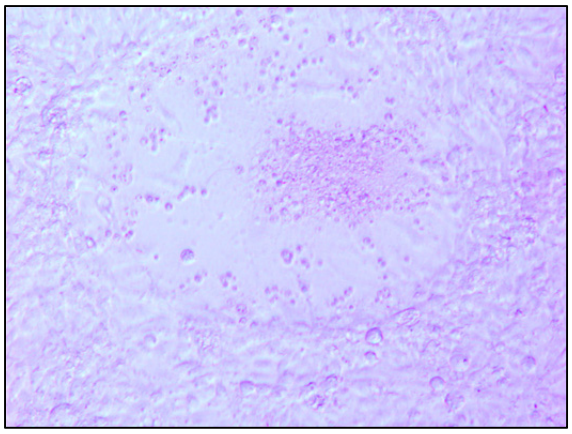

Figure 4. Atlantic salmon Aquareovirus on BF-2 cell line.

Orthoreoviruses are naked viruses with icosahedral symmetry of the capsid, but appearing spherical in shape. The protein capsid is organized in two concentric layers with an outer diameter of approximately 80 nm, and it surrounds ten linear dsRNA segments that make up the viral genome.

Mammalian orthoreovirus (MRV) has been a model virus for the study of viral infection mechanisms in general, particularly for viral entry [49], genome replication and formation of viral factories [50]. MRV infects the intestine and through hematogenous route spread to the CNS [51]. Recently MRV has been strongly associated with celiac disease, being the virus capable of disrupting intestinal immune homeostasis and initiate loss of oral tolerance and immunity to the dietary antigen. Despite being apparently non-virulent and being successfully cleared from the infected host, reoviruses appears to promote immunopathology [52].

Member viruses of the genus *Orthoreovirus* are widespread and include viruses found in cattle, sheep, swine, humans, nonhuman primates, bats, birds, and fish (Fig.5). However, most of these infections are not associated with any significant clinical disease, with orthoreoviral infections of poultry being exceptions. Recently, also fish orthoreoviruses have been found to be the causative agent of disease.

Modern poultry production share many similarities with aquaculture in terms of confinement of large numbers of homogenous animals, high turnover in the production, etc. Avian reoviruses (ARV) have been associated with a variety of disease conditions in poultry. These include enteric and respiratory diseases, myocarditis, hepatitis, and the so-called stunting/malabsorption syndrome. However, a direct link between the presence of the virus and disease has only been conclusively demonstrated for viral arthritis syndrome (also known as tenosynovitis), which is characterized by swelling of the hock joints and by lesions in the gastrocnemius tendons [53].

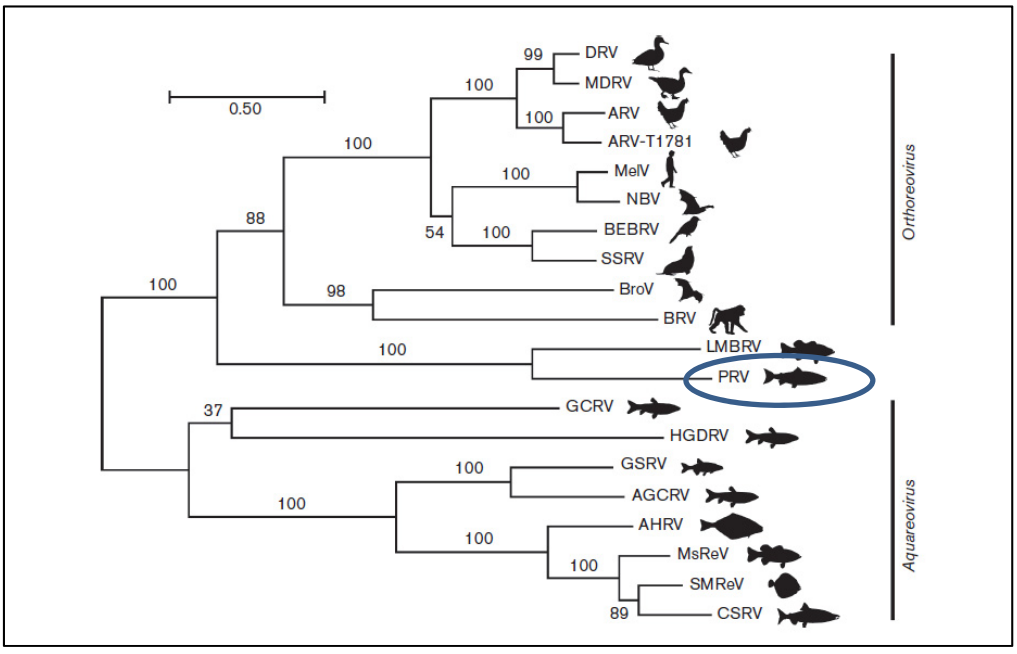

Figure 5. Phylogeny of *Orthoreovirus* and *Aquareovirus* genera , based on genomic segment L3. Figures modified from Sibley et al.2016 [54].

Within *orthoreovirus*, PRV has been accepted as a new species infecting fish [39, 55–57]. Moreover, a virus putatively termed Largemouth Bass reovirus (LMBRV) has recently been described and associated with disease in Largemouth bass (*Micropterus salmoides*) [54].

In human and veterinary virology, viruses have classically been addressed and characterized if they cultivable in cell cultures. The introduction of NGS and sequence independent single primer amplification has recently led to the discovery of a number of nucleic acid sequences originating from viruses which are yet to be classified. The magnitude of the number of these new viruses will have a large impact on the taxonomic order we use today, and furthermore, they necessitate an increased focus on assessment of their biological relevance in farmed animals.

Piscine orthoreovirus (PRV)

PRV is a non-enveloped virus with a segmented double-stranded RNA genome enclosed in a capsid with two concentric protein layers [55, 58]. Despite the low nucleotide and amino acid conservation between PRV, MRV, ARV and GCRV at the genome level, a remarkable strong conservation of protein secondary structure across genus lines of has been predicted. These findings illustrate the conservation of secondary structure over the primary sequence, reflecting the importance of structural features for functionality, where even minor alterations would be deleterious [55].

i) GENOME. Markussen, Takano, and Kannimuthu [55–57] have described the genome of PRV-1, PRV-2 and PRV-3 subtypes. The genome of PRV consists of 10 segments grouped in three sizes; L (long), M (medium) and S (short). There are three L and M and four S segments. The secondary structure of the proteins appear very conserved, and therefore the MRV is contained as the prototype virus [55]. An overview of the segments, the expected protein encoded and its function is given in table 1.

Table 1. PRV gene segments, encoded protein and predicted function.

Segment	Protein	Function
L1	λ 3	RNA-dependent RNA polymerase
L2	λ 2	Guanylyltransferase, methyltransferase
	P11	p11: hypothetical protein
L3	λ 1	Helicase, NTPase, RNA triphosphatase
M1	μ 2	NTPase, RNA triphosphatase, RNA binding
M2	μ 1	Outer capsid protein, membrane penetration
M3	μ NS	Non-structural protein
S1	σ3	s3: outer capsid protein, zinc metalloprotein
	P13	p13: cytotoxic, integral membrane protein
S2	σ 2	s2: Inner capsid protein, RNA binding
	P8	p8: hypothetical protein
S3	σ NS	Non-structural protein, involved in virus inclusion formation
S4	σ 1	Cell attachment protein

ii) VIRAL PROTEINS. The PRV genome consists of 10 segments with putatively 13 ORFs or more. The proteins translated from L segments are involved in viral replication. L1 encodes for λ3, the virus' RNA-dependent RNA polymerase (RdRp) responsible for viral transcription and replication. L2 encodes for 2 proteins; λ2 the capping enzyme contributing to generate the 59-terminal cap, on virally encoded mRNAs. L2 segment putatively encodes also for P11, a protein whose function is not described yet. L3 is predicted to encode the core capsid shell λ1 protein. M1 encodes for μ2, which binds both ssRNA and dsRNA and interacts with the RNA polymerase. M2 encodes for μ1 the major outer capsid protein. M3 encodes for non-structural protein μNS; involved in the formation of viral factories where virion assembly occur in the erythrocyte cytoplasm [59]. S class segments encode for both outer (S1 – σ3) and inner capsid (S2 - σ2), and the cell attachment protein (S4 – σ1). S3 encodes for non-structural protein σNS; this protein binds nucleic acids in nuclei or cytoplasm of infected cells. S1 and S2 have both an additional ORF encoding respectively for P13 and P8 (putative). P13 has cytotoxic activity [60]. Interestingly, this protein is not fusogenic, and PRV does not appear to have FAST protein encoded by its genome [60]. For P8 no function has been described.

iii) CAPSID. The theoretical model to solve the architecture of viral capsids relies on icosahedral symmetry. This solid is constituted of 20 triangular facets. Each triangular facet is constituted of viral capsid proteins. The ratio between the number of capsid protein and facet is defined as Triangulation number (T) [61]. Orthoreoviruses has 2 concentric capsids (Fig.6); these structures are essential in the attachment, cell entry, and viral replication processes. The inner capsid layer of orthoreovirus particles has an internal diameter of approximately 50–60 nm with T=2* icosahedral symmetry composed of 12 pentameric dimers for a total of 120 capsid proteins. The T=2* symmetry is a nonofficial appellation, to be rigorous the capsid has a T=1 symmetry with each unit composed of a homodimer. The inner capsid is composed of proteins λ1, σ2, λ3 and μ2. The outer capsid has a T=13 icosahedral structure, which is composed of 12 pentameric and 120 hexameric capsomeres for a total of 780 capsid proteins. It is composed of σ1, μl, and σ3. The vast majority of the capsid is constituted by μl, and σ3. In the outer capsid, the symmetry is interrupted by λ2, which connect both capsid and form the "turret" like structures which are conserved between ARV and MRV [62]. The proteins of the PRV virion have recently been described and partially functionally characterized (co-precipitation assays) [58, 59]. Mature virions lack a lipid envelope.

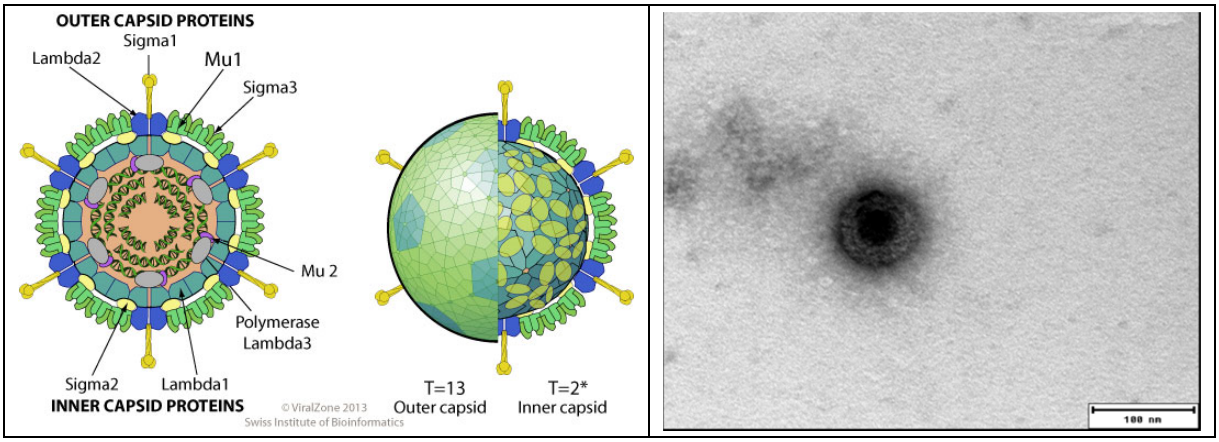

Figure 6. On the left schematic orthoreovirus structure (ViralZone.Expasy); on the right Electron Microscopy (EM) picture of PRV-3 purified from infected blood of Rainbow trout. The EM picture display to electron-dense layers representing inner and outer capsids

iv) SUBTYPES. The term "subtype" is used as the different viral types within *piscine orthoreovirus* do not fulfill the requirements provided by ICTV for a new viral species, yet these viruses have different genetic and antigenic properties, furthermore, a preferential ecological niche has been described for each PRV-type. Currently, three subtypes of PRV have been genetically and phenotypically characterized. These include PRV-1 [55], a highly prevalent virus in the sea phase of farmed Atlantic salmon in Norway; PRV-1 is the causative agent of heart and skeletal muscle inflammation (HSMI) in Atlantic salmon [39]. A second subtype PRV-2 has been demonstrated to be the causative agent of erythrocytic inclusion body syndrome (EIBS) in Coho salmon (*Onchorhynchus kisutchi*) [57]. Finally, a third subtype PRV-3 [56] was initially associated with new disease in Rainbow trout [45, 63]

where the causative relationship recently has been proven (paper 3 - Vendramin et al., in press). During the initial descriptions of this virus, PRV-3 has been temporarily named as PRV-like and PRV-Om [45, 63]. It has to be noted that the subtype classification of PRV (PRV-1, PRV-2, and PRV-3) followed the time of discovery rather than phylogenetic relationship. In fact, PRV-1 is closer related to PRV-3 than to PRV-2 [56]. Segment L1 encoding for the RNA-dependent RNA polymerase appears to be the most conserved segment sequence wise and has therefore been selected as the target for qPCR for diagnostic and surveillance [45, 57, 64, 65]. The genetic diversity among PRV subtypes makes it difficult to have a qPCR target for a "pan-PRV test" and only subtype-specific assays are implemented in diagnostic laboratories. As a practical consequence, screening programs and targeted surveillance aiming to assess the prevalence of different PRV-subtypes rely on multiple testing. Importantly, segment S1, which encodes for the outer capsid protein σ3, is the most variable segment within PRV-subtypes and therefore has been chosen as the target for conducting phylogenetic studies [55, 66, 67]. Notably, for PRV-3, sequence analysis conducted so far for S1 segment support the presence of 2 different clades (Fig.7). One clade includes the original isolate detected in Norway in 2013; the other clade includes the PRV-3 isolates detected in Scotland, Denmark, Germany, Italy and a series of sequences deposited in Genbank [68] described in Chile [56]. The homology between the PRV-3 sequences detected in Chile and in Europe suggests an epidemiological link. Trade of live fish eggs might provide an explanation.

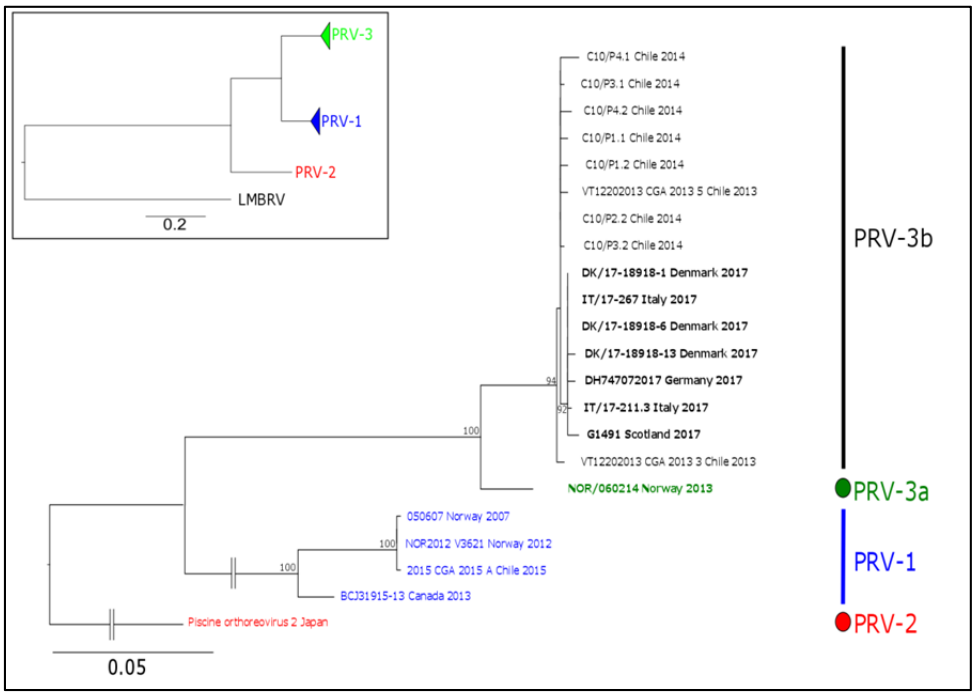

Figure 7. Phylogenetic analysis of partial nucleotide sequence of S1 segment of various PRV-3 isolates. The inner box represents the tree topography drawn in scale 0.5. The tree shows two PRV-3 clades, PRV-3a, and PRV-3b with strong support [56].

v) REASSORTMENT. Segmented RNA viruses are prone to reassortment. This is a biological process where two or more viruses with segmented RNA genome (define as parental strains), infect the same host cell and exchange RNA segments. The progeny from the co-infection harbors genetic material from both parental strains, and potentially with higher fitness [69]. Successful reassortment requires compatibility of packaging signals and capsid protein-RNA interaction; nonetheless, reassortment might produce a viral progeny with lower fitness deemed to be outcompeted by parental strains in the ecological niche. Reassortment is already described for segmented RNA virus in fish such as the case of beta- nodaviruses, IPNV and ISAV [70–73]. In the case of PRV, reassortment within PRV-1 subtype has already been observed [67], among the 60 sequences aligned obtained from wild, hatchery-reared (for re-stocking purposes) and escaped fish, 6 isolates displayed reassortment when comparing S1 and S2 clustering; 6 isolates displayed reassortment when comparing S1 and S4 clustering. Whether successful reassortment between PRV-1 and PRV-3 can occur, is unknown. PRV-1 and PRV-3 infect their specific host Atlantic salmon and Rainbow trout respectively, which are farmed in close proximity to each other in Norway and Chile. Furthermore, during the course of this project, PRV-1 has been repeatedly detected in wild native Atlantic salmon returning to spawn in the river in Denmark where PRV-3 is prevalent in Rainbow trout farms. The co-existence of the two different hosts and related PRV-subtypes create an epidemiological opportunity for reassortment to occur, and this should be further monitored and investigated.

PRV epidemiology

The prevalence and distribution of PRV are two folded, with virus presence in different fish species and related stocks (wild and farmed) on the one hand and the occurrence of the disease outbreak on the other.

It is important to underline that, the mere detection of the viral RNA acquired through extensive screening based on qPCR does not provide evidence of disease outbreak. Likewise, the observation of histopathological heart inflammation or anemia does not provide evidence for PRV-mediated disease outbreak, as these findings can have many causes. Other common viral pathogens such as PMCV (associated with CMS) and SAV (causing PD) cause heart pathology to be differentiated from HSMI [74].

The orthoreoviruses are, in general, ubiquitous in their respective niches, and so are PRV. Each of the three currently described PRV subtype has been demonstrated under experimental conditions, to be the causative agent of a specific disease:

1) PRV-1 is the causative agent of HSMI in Atlantic salmon [39]
2) PRV-2 causes EIBS in Coho salmon [57]
3) PRV-3 causes pathologic lesions in the heart in Rainbow trout (Vendramin et al., in press).

Furthermore PRV has been associated with other clinical manifestations in salmonids; PRV-1 has been associated to Jaundice syndrome in Chinook salmon (*Oncorhynchus tshawytscha*) [75], PRV-3 is associated with Jaundice syndrome in Coho salmon [66]; finally PRV-1 has been associated with the development of melanized spot in Atlantic salmon fillet [76]. These findings will be further discussed later in the thesis.

PRV-mediated diseases are not a notifiable disease, and therefore official figures are not available. The level of resolution for disease mapping is limited to reports provided at the country level.

1) PRV-1 becomes ubiquitous in Atlantic salmon farmed in Norway during the sea phase [58, 65] estimating that prevalence of PRV-1 in farmed Atlantic salmon late in the production cycle is close to 100%. In the North-Atlantic the virus is also detected in farmed Atlantic salmon in Faroe islands [77, 78], Iceland [79], Ireland [80] Scotland [81, 82] and France (Bigarré and Boitard personal communication). In the Pacific ocean, PRV-1 has been detected in Chinook salmon, and Atlantic salmon farmed in British Columbia, Canada [75, 83, 84], Atlantic salmon, Coho salmon, and Rainbow trout farmed in Chile [66, 85].
   The detection of PRV-1 occurs also in wild stocks of Atlantic salmon and Sea trout (*Salmo trutta*) in Norway [67, 86], in Germany [87] in Canada [83, 88] and in wild stock of Chinook, Coho, Pink salmon (*O. gorbuscha*) and sea running Rainbow trout (Steelhead) (*O. mykiss*) in Alaska and Washington [89]. The estimated prevalence in Norwegian wild fish is 10% in Atlantic salmon and 3% in sea trout. In this project (manuscript 4 - Vendramin et al., 2018) we have investigated the presence of PRV-1 in wild Atlantic salmon stocks in the northern Atlantic. Although with lower prevalence compared to the assessment conducted in Norway, PRV-1 has been detected by q-PCR and partially sequenced (S1 segment-σ3 protein) from Denmark, Sweden, Ireland, and the Faroe Islands. The virus has not been detected, although

investigated, in wild salmonid fish in France, Belgium, and Greenland. Finally, PRV-1 detection in wild non-salmonid marine fish has been reported in Capelin (*Mallotus villosus*) Great silver smelt (*Argentina silus*) Atlantic horse mackerel (*Trachurus trachurus*) Atlantic herring (*Clupea harengus*) [90]. Predation of PRV infected escaped Atlantic salmon from wild fish such as Cod (*Gadus morhua*) has been speculated as the source of transmission from farmed to wild fish [91].

In surveillance investigation of archive samples, PRV-1 has been suspected in samples collected in 1977 from steelhead, and Atlantic salmon collected in 1987 in British Columbia [88].

HSMI outbreaks are reported in Norway (Fig. 8) [92], Scotland [81, 82], Canada [93], Chile [66] and France (Boitard personal communication), the disease was described for the first time in 1999 [94]. HSMI is not listed disease, and it is expected that the number of outbreaks is higher than what here reported.

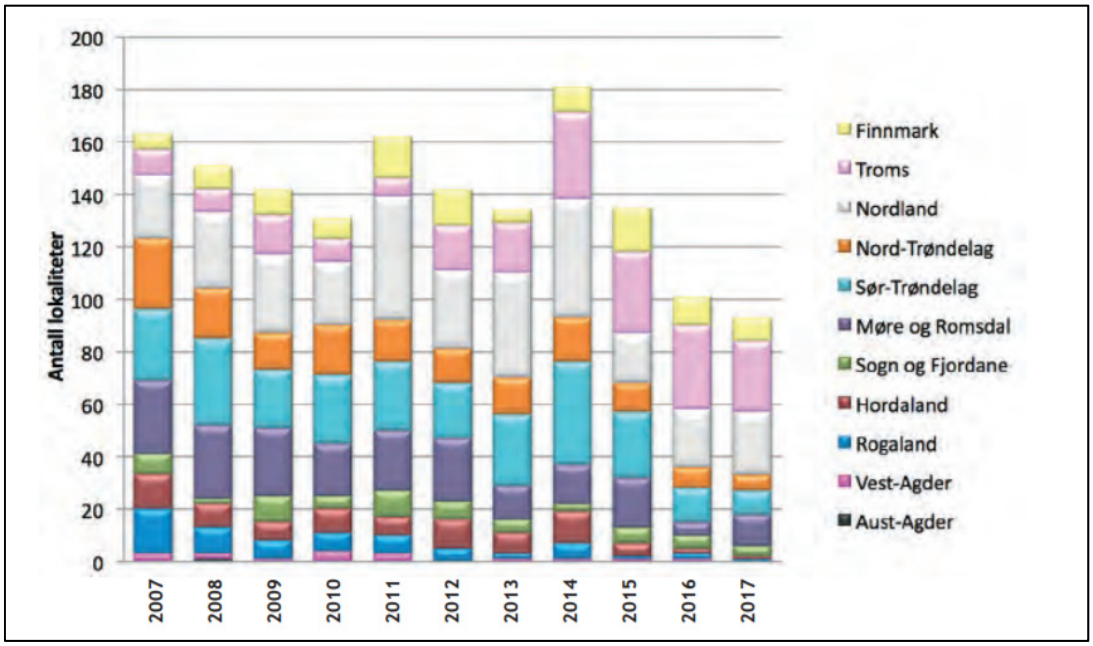

Figure 8. Number of HSMI outbreak reported to National Veterinary Institute in Norway in 2007-2017 From Fish health report 2017 [92]

2) To the best of the author's knowledge there have not been screening programs for PRV-2; therefore the only information related to PRV-2 rely on the reports of EIBS outbreak available. This disease is associated with severe economic losses in Coho salmon (*Onchorhynchus kisutchi*) in Japan. The disease was first described in 1977 in Rainbow trout [95] and then in 1982 in juvenile chinook salmon (*O. tshawytscha*) reared in a freshwater hatchery in Washington, USA [96]. A number of salmonid of the *Onchorynchus* genus has been tested experimentally for development of inclusion body in the red blood cells and reduction of hematocrit (signs of EIBS) [97]. After experimental exposure to blood obtained by clinically EIBS affected fish: Chum salmon, Coho salmon, and Masu salmon were equally susceptible. Interestingly also Rainbow trout showed susceptibility to this challenge but to a lower extent [97]. Finally, Rodger and Richards reported inclusions bodies in red blood cells of Atlantic salmon farmed in Ireland [98]. PRV-1 infection is also associated with intracytoplasmic inclusions in red blood cells [99, 100]; these findings were not confirmed by qPCR methods. Furthermore, in a proportion of samples collected from the sea phase histopathological findings of cardiomyopathy were described, leaving speculation that it was PRV-1 and HSMI detected in Ireland rather than PRV-2/EIBS.

3) PRV-3 has recently emerged as a pathogen in farmed salmonids. PRV-3 was initially described as "PRV-related virus" while investigating a new disease in Rainbow trout causing HSMI-like pathology [45]. Closely related sequence originates from Rainbow trout affected by the idiopathic syndrome of Rainbow trout (ISRT) [56] from 2014 and Coho salmon [101] in Chile. In 2017 the increased awareness for this new virus has led specific screening programs. As a result, PRV-3 has been detected in Germany [87], in Scotland and in Denmark in relation to a clinical disease outbreak in Rainbow trout [56]; the virus has also been detected surveillance samples from asymptomatic samples of brown and Rainbow trout in Italy [56] (Fig. 9).

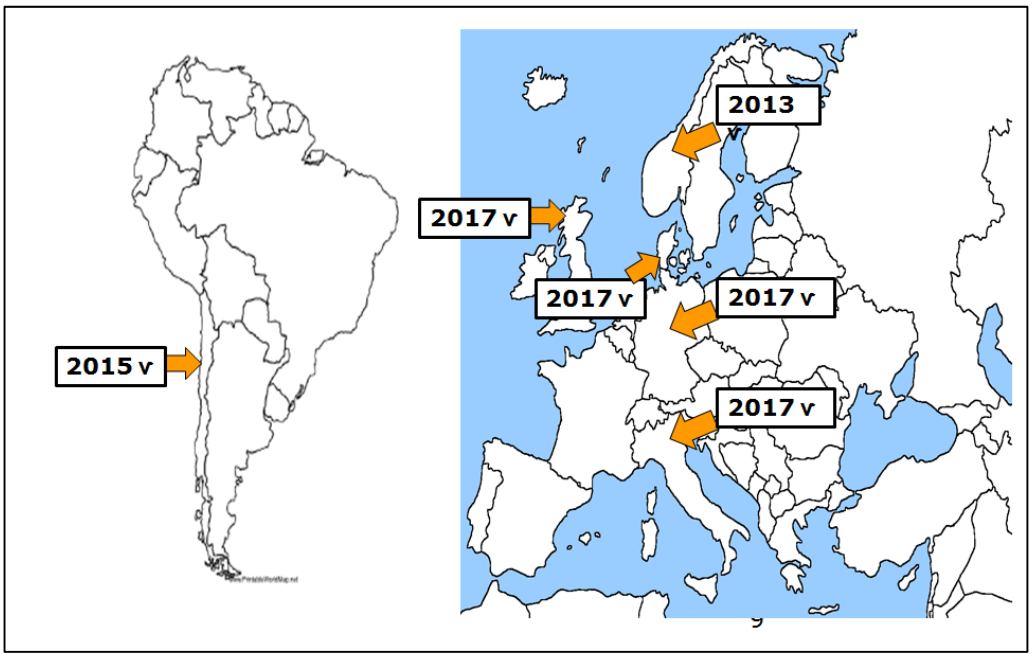

Figure 9. Distribution of PRV-3 worldwide

The detection of PRV-3 in relation to the occurrence of clinical disease outbreaks has posed the question if and possibly when the virus has been introduced to the country. In a small survey conducted in 2016 on five Danish flow-through fish farms, selected as high risk for introducing the infection, the virus was not detected. In 2018, an extensive survey in Denmark (Henriksens Fund Project 2018-03-06-163320) was conducted to assess the PRV-3 prevalence in the country. A panel of fish farms was selected, comprising both flow-through and RAS farms, and representing different "farm clusters" including re-stocking facilities (farming brown trout), broodstock farms, Nursery/juvenile farms, grow out farms. From each farm 30 fish were collected. Organs were homogenized and pooled (five specimens per pool). Samples were tested by qPCR [45]. The results obtained showed a significant change in the PRV-3 prevalence in 2018, with 35 out of 52 farms were positive (Fig. 10; Table 2).

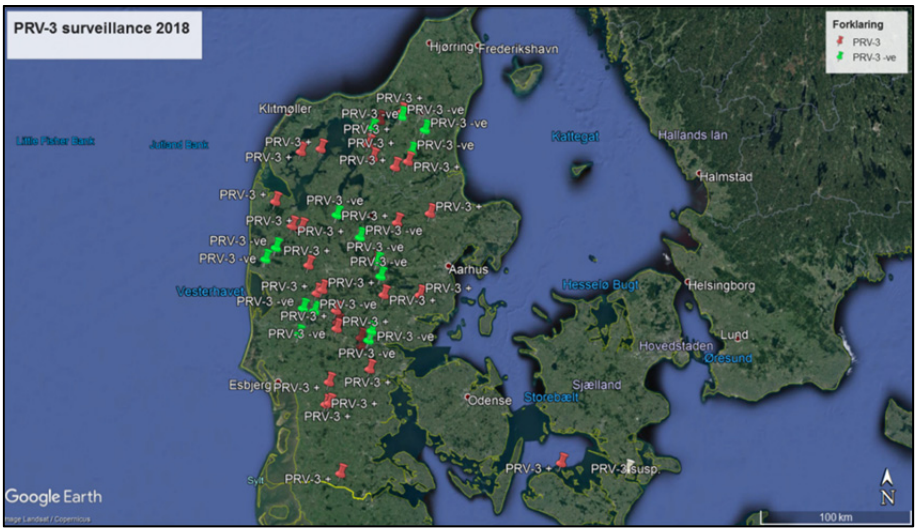

Figure 10. PRV-3 mapping in Danish fish farms in 2018. Red pins indicate fish farms tested positive for PRV-3; green pins indicate fish farms tested negative; white pin indicates farm suspected.

Table 2. PRV-3 prevalence in Danish farms.

Farm type	Farmed species	# farm tested	# PRV-3 +ve farm	Presence of disease
Re-stocking	Brown trout	3	3	0
Broodstock	Rainbow trout	21	10	0
Nursery	Rainbow trout	13	9	0
RAS Grow out	Rainbow trout	15	13	10

These results indicate that Brown trout is a susceptible species to PRV-3 infection and represents a potential reservoir of the virus in the wild, corroborating the finding of Brown trout PRV-3 positive in Italy [56]. With regards to the Danish farming industry, the infection is present in the broodstock farms, potentially allowing egg-associated transmission of the virus in the downstream production (nursery and grow out). It is not known if PRV can transmit vertically, but the low prevalence of PRV-1 in fresh water facilities in Norway indicate that it is not a common route of transmission. Importantly, 52% of the broodstock farms tested negative. These farms will be included in the health screening program to assess whether it is possible to maintain a PRV-free production.

PRV transmission routes

For PRV-1 and PRV-3, the horizontal transmission has been demonstrated under experimental conditions in cohabitation challenges [39, 63], whereas for PRV-2 field evidence show horizontal transmission during epizootics [57]. Reo is an acronym for respiratory, enteric, orphan, but refers to the portals of entry and target organs for mammalian reoviruses. Although fish in seawater drink continuously, due to the necessity to have a stable electrolytic balance, the volume of water passing over the gills is much bigger. Furthermore, the base of fins has been found to be possible portals of entry for rhabdoviruses [102]. Together, there are several portals of entry for fish viruses; oral, respiratory, fin-base, and it is difficult to pinpoint this in detail for the particular virus. It is also possible that this property may vary between strains of the virus, possibly making studies of this subject difficult.

For PRV-1 in Atlantic salmon, the portal of entry of the virus still has to be thoroughly investigated. Under the experimental condition, administration of PRV through anal intubation of the Atlantic salmon gave infection kinetics comparable to the one of i.p. injected fish. Histopathological examination further demonstrated that fish infected through the anal route develop HSMI [103].

For PRV-3 in Rainbow trout, the infectious aetiology and horizontal spread were observed during the first described disease outbreak [45]. In that case, three hatcheries and two grow-out farms epidemiologically connected by sourcing eggs from a common producer experienced the PRV-3 disease.

A longitudinal study conducted under farming conditions in Norway showed that, the progeny tested PRV negative, despite a high PRV-1 prevalence(>90%) of broodstock, and that the vast majority of the infection occurs after seawater transfer [65, 104]. These findings and the epidemiological picture of HSMI in Norway, indicate that vertical transmission route is not common or perhaps unlikely to occur in that management settings. It cannot be excluded that egg-associated transmission through contamination of eggs and milt might occur, highlighting the importance of proper egg disinfection procedures, which rely on sufficient time of exposure and concentration of the disinfectant [105].

The interaction between wild and farmed fish population in the aquaculture system imply pathogens and related disease transfer in both directions. An epizootic in the net pen could cause shedding of a pathogen in a sufficient level to make wild population contract the infection, and vice-versa; infected wild fish might shed and thus expose a pathogen to naïve fish in the farm.

In the case of PRV, there is an ongoing debate on the impact of this infection on wild salmonid populations. Recent findings associating PRV-1 infection and Jaundice syndrome in Chinook salmon have raised awareness of the impact of sea cage aquaculture in British Columbia [75]. There is scientific evidence that wild salmon returning to spawn has a certain prevalence of PRV (8-10%) [67, 86] and time at sea appear to be important risk factors in contracting the infection [67, 86]. Studies conducted in Norway and British Columbia observe that PRV prevalence in wild fish is not affected by the presence of Atlantic salmon net pen farm, nor by the occurrence of HSMI outbreak in such farms, therefore, concluding that prevalence in wild fish is not associated to salmon farming [67, 86, 88]. However, the wild salmon returning to the Norwegian coast after feeding in the ocean

do not go directly to its home-river, but the majority is moving along the coast until the correct river is found [106]. This gives ample possibilities for contact between farms and adult salmon, and difficult to try to link such contacts to the appearance of HSMI or not in particular farms. Salmon smolts swim straight out to the big seas but may pass by farms after leaving the river outlet. Sea trout, on the hand, move along the coast, and are hypothetically, more exposed than salmon to agents from farms. Furthermore, the commonly observed identity of S1 nucleotide sequences between PRV originating from farmed and wild fish indicate an epidemiological link.

Our findings (manuscript 4) showed PRV in wild Atlantic salmon in Denmark, where currently no Atlantic salmon farming in the sea is present.

The question on when in the lifecycle and where wild fish get infected is still open. One option is that PRV is shared on feeding grounds, i.e., Faroese Islands or Western Greenland [67], another possibility is that fish get infected when returning as an adult to the river of origin to spawn while transiting through the farming areas.

The limited data available show that PRV prevalence in smolts entering the ocean is smaller than wild salmon returning to spawn that in a fjord in western Norway [86]. During this study, we have investigated wild salmon in West Greenland (N=30 in 2017), but no PRV was detected in those samples. This could indicate that the prevalence of the virus in the wild fish, i.e., without contact to farmed fish, is not high. Although only 30 fish were tested, it could indicate that it is more likely for wild fish to be infected after transiting through the farming areas in the north Atlantic.

Notably, in the survey of wild salmonids conducted in this project (manuscript 4), PRV-1 was detected in wild Atlantic salmon caught in the feeding ground north of Faroe Islands. The prevalence varied significantly from 2009 (5 out of 88; 5,68%) to 2011 (33 out of 76, 43,42%), nevertheless these data suggest that wild salmon can get infected also in their journey to reach the feeding ground, after they left the river of origin.

PRV replication

Viruses need to hijack different components of the cell machinery to accomplish a productive replication cycle. Despite considerable efforts, it has not been possible so far to propagate PRV in permanent cell lines *in vitro* (Niels Bohls and Espen Rimstad personal communication).

It is assumed that PRV replication closely follows the one of MRV (Fig. 11), a widely studied model for orthoreoviral replication [50].

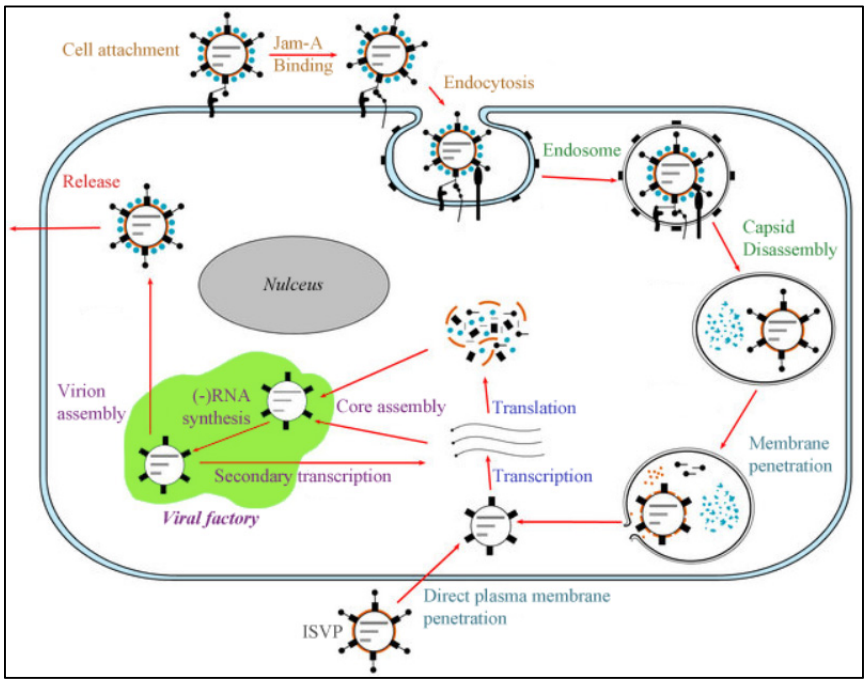

Figure 11. Model of the mammalian orthoreovirus replication cycle and known host proteins and cellular machinery that participate in each replication step. Pictures obtained from Sahin et al., 2013 [107]

During orthoreovirus replication 3 different "phenotypic" conformations of the virus are involved. The mature MRV virion attaches to the surface membrane of the target cell through the receptor-binding mechanism, which involves junctional adhesion molecule-A (JAM-A) receptor and σ1 surface protein [108]. Neurotropic variants of the virus use a different cellular receptor [109], and the MRV σ1 surface protein is capable of binding to erythrocytes through yet different cell surface molecules; *orthoreovirus* binding of erythrocytes can agglutinate these [110]. In the case of PRV, it can be speculated that JAM-A is not the main receptor, being poorly expressed in erythrocyte. The receptor attachment trigger endocytosis by the host machinery and internalization, initially in the *caveolae* and then into endosomes [111]. The pH decrease accompanies maturation of the endosomes where virion are transformed into infectious subviral particles (ISVP) through the cleavage of σ3 and μ1 [112–115]. During the virus entry, the μ1 is auto-cleaved to facilitate core delivery [116, 117]. This autolytic cleavage site residue at N-terminal N42P43 is conserved between PRV and other orthoreoviruses [55]. In MRV, the second cleavage of the C-terminal μ1 generates the φ fragment, which is linked to optimal infectivity [118]. The third domain of MRV μ1 protein consists of five α helices and contain a protease cleavage site for δ-φ cleavage [119]. The differences in this second cleavage site and the generation of differently sized δ and φ fragments

observed between PRV and MRV could be linked to structural differences that were observed in this region; the loop region aa 72–96 of MRV µ1 protein is shown to contribute to the stabilization of the capsids [120]. PRV, avian orthoreovirus (ARV), and aquareoviruses all lack this loop [121], which could be linked with the evolutionary adaptation of the virus to different host species. The transformation from virion to ISVP can also occur outside of the endosome environment, such as partial intestinal digestion; partial digestion of the external capsid of MRV did not inhibit infection [122]. ISVP have the opportunity to enter the cell by forming pores in the host cell membrane through µ1 protein [49]. ISVPs can penetrate the endosomal membrane through pores formed by the cleaved N terminal domain of µ1 and transform into core particles which have lost the σ1 compared to ISVPs and are deposited into the host cell cytoplasm [118]. The σ3 also travels to the cytosol where they act as RNA binding molecules and bind to µ1 [119].

Core particles are not infective, they are on the other hand the initial machine for viral mRNA production [123]. During virus replication, positive-sense single-stranded (+)RNAs are packaged into procapsids, where they serve as templates for dsRNA synthesis, forming progeny particles containing a complete equimolar set of genome segments. How the (+)RNAs are recognized and stoichiometrically packaged remains uncertain. The assembly of new virion occurs within viral factories (Fig. 12), organized in the perinuclear cytoplasm by µNS protein [59].

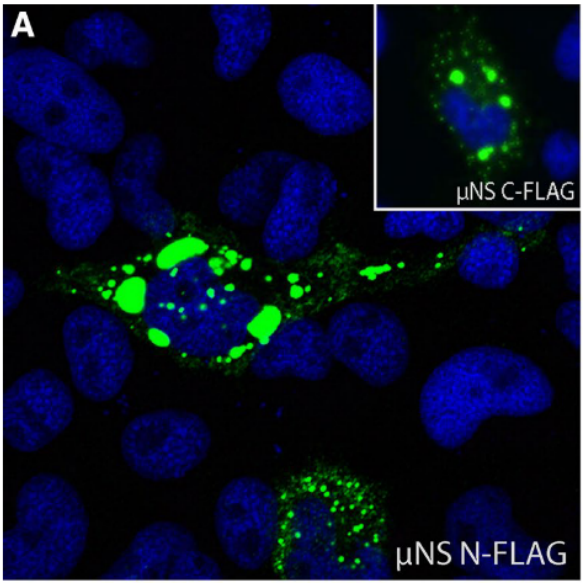

Figure 12. PRV-1 µNS protein expression of transfected EPC cells. µNS organize in the cytoplasm to form a vacuolar structure resembling viral factories. Picture from Hanne Haatveit et al.,2016. [59]

PRV infects and replicates in red blood cells [99]. Notably, fish erythrocyte are nucleated cells capable of protein synthesis [124] which decrease with increased age of the cell [125]. During infection in Atlantic salmon, PRV-1 RNA segments are transcribed homogenously (S. Malik, personal communication 2018). Fish erythrocytes are capable of raising innate immune responses during PRV infection and have been shown to express immune genes related to IFN and MHC class I [99, 126].

Pathogenesis of PRV infection

PRV was discovered in 2010 [64] thanks to the implementation of next-generation sequencing combined with mathematical and bioinformatical modelling [40]. Piscine orthoreoviruses are widely spread in the ecological niches of farmed salmonids. The virus has withstood cultivation in cell cultures, and this restricted capacity of testing viral viability has significantly complicated the understanding of host-pathogen interaction, which often is constrained to detection of various qPCR analysis. In order to overcome this limitation, the propagation of the virus *in vivo* and the purification by gradient ultracentrifugation has yielded pure viral particles to be included in experimental trials in fish. This approach has led to demonstrate a causative relationship between the three PRV subtypes and disease/histopathological lesions in 4 fish species.

- PRV-1 is the causative agent of HSMI in Atlantic salmon [39]
- PRV-2 causes EIBS in Coho salmon [57]
- PRV-3 cause PRV-3 disease with heart pathology in Rainbow trout (Vendramin et al., in press) and brown trout (Vendramin et al., under preparation)

HSMI is an infectious disease affecting farmed Atlantic salmon usually five to nine months after sea transfer. The mortality due to HSMI has been reported to be as high as 20 %, while the morbidity is close to 100 % in affected cages [127]. However, usually the mortality rate is much lower and scores only a few percents points over the production cycle. Fish surviving HSMI outbreak have cardiac lesions for months after an outbreak. Characteristic, but not pathognomonic, histopathological findings of HSMI are epi-, endo- and myocarditis, myocardial necrosis, red skeletal myositis and necrosis (Fig. 13) [94]. Mortality is likely to be the consequence of the circulatory failure, including the reduced colloidal properties of the blood, which leads to oedema; heart reduced efficiency due to inflammation, and reduced capacity of exchanging oxygen of the erythrocyte.

Other infectious diseases common in the seawater phase, such as CMS [128] and PD [129], have to be considered in the differential diagnosis of HSMI [74], since they display a similar pathological picture.

PRV infection in Atlantic salmon induces an innate antiviral immune response in its major target cell, the erythrocyte [126], thus this response can be measured in any vascularized organ. The innate antiviral response elicited by PRV has been described in various organs such as the spleen, head kidney and heart [58, 130]. The up-regulation of IFN and IFN mediated genes initially follows the viral RNA load [100, 131]. The inflammatory response in cardiac tissue of Atlantic salmon is associated with increased level of the CD8+T cell transcripts [58, 132] implying that CD8+ cytotoxic cells are involved in the mechanism that PRV-1 trigger to induce HSMI [132]. Under the experimental condition, PRV-1 infection in Atlantic salmon develops into a persistent infection. Typically 4 weeks after injection, the viral RNA reaches its peak in the IP injected shedders, and 2 weeks after (6 weeks post challenge-wpc) in the cohabitants. In an experimental trial conducted in Canada [133], PRV was not transmitted from shedders to cohabitants; at 45,47,63 wpc, although it was still possible to detect a significant load of viral RNA in the shedders. Furthermore, it has been shown [58] that soon after the PRV-1 viral RNA peak (4 wpc) all PRV-1 proteins are no longer detectable by flow cytometry, however, a cleaved fragment of µ1 can be detected in Western Blot. Finally, a bead-based immunoassay developed to detect specific antibodies to PRV-1 proteins (µ1c and µNS), [134], showed that specific antibodies are detectable 6 wpc (peak of viraemia 4 wpc) and persist until at least 10 wpc. These findings provide important indications of the shedding phase of the virus, which is likely to be restricted to the phase peak of the viraemia.

The role of PRV-1 in HSMI has been questioned [133]. The causative relationship between PRV-1 infection and HSMI development was demonstrated in a challenge trial where purified PRV-1 particles were used [39]. An experimental challenge, using a PRV-1 isolate from British Columbia gave viremia in IP injected Atlantic salmon and Sockeye salmon (*Oncorhynchus nerka* Walbaum), could transfer to cohabitant fish of the same species, but no histopathological findings related to HSMI nor induction of interferon (IFN) pathway were observed [133]. This has raised interest in virulence markers, linked to the genetic variation of PRV-1 isolates [55, 83].

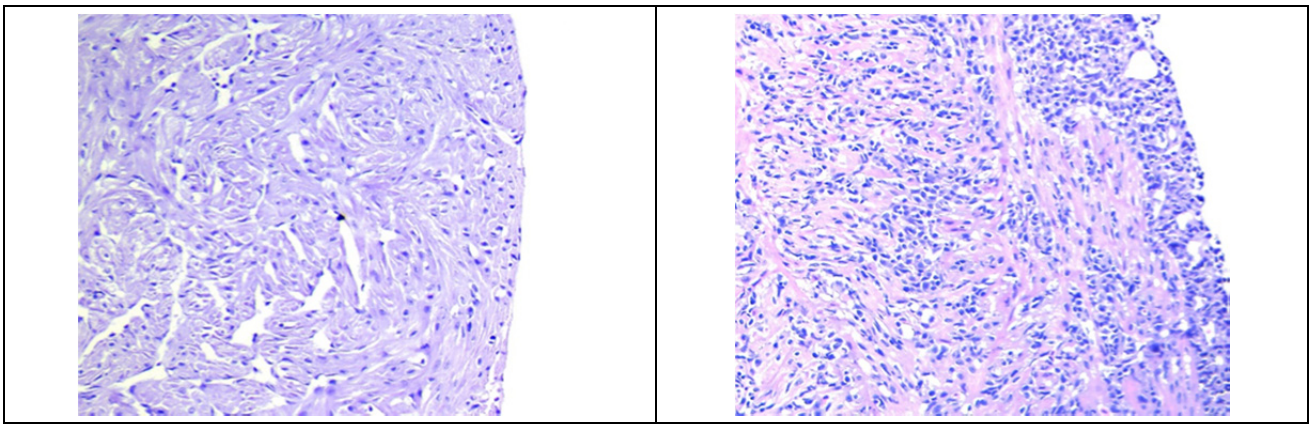

Figure 13. On the right, Atlantic salmon heart tissue negative control. On the left severe inflammation of epicardium and compact and spongious myocardium, from PRV-1 infected fish with HSMI. Pictures from Anne Berit Olsen[131]

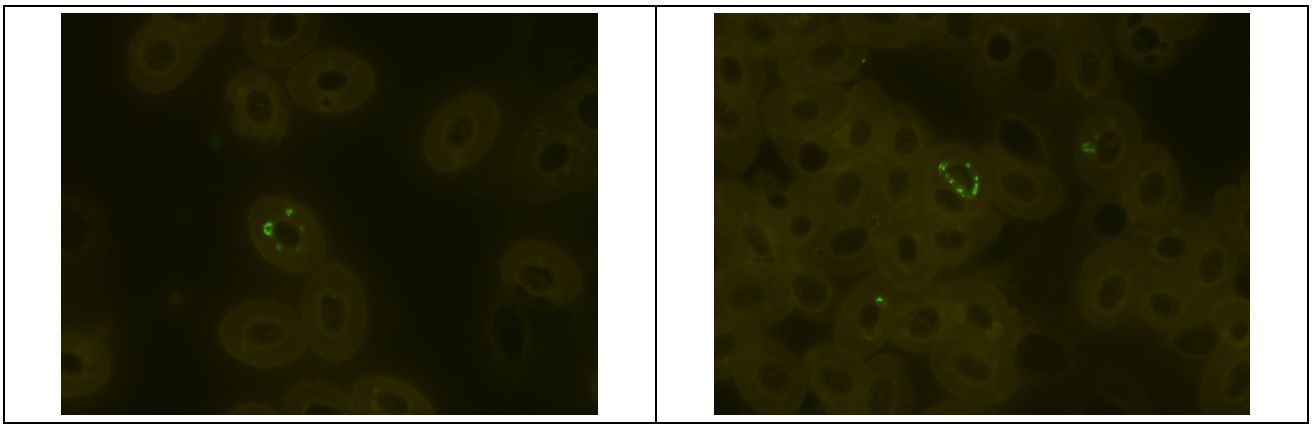

Figure 14. Intracytoplasmatic, perinuclear immunofluorescent staining of PRV σ1 antigen in Atlantic salmon erythrocytes infected with PRV.[131]

To conclude, HSMI is a disease of Atlantic salmon, caused by the host immune response to PRV-1 infection.

Table 3. Resume of the pathogenesis of HSMI in Atlantic salmon

PRV-1 infection in Atlantic salmon cohabitants					
Time	**2 w.p.c.**	**4 w.p.c.**	**6 w.p.c.**	**8 w.p.c.**	**10 w.p.c.**
PRV RNA Kinetics		Peak of PRV RNA (L1 segment)	Plateau phase of PRV RNA (L1 segment) (probably lifelong, lasts at least until 57 wpc, common in broodstock)		
PRV protein kinetics in RBC	Non detected	Detected	Undetectable		
Histopathology	No lesion	No lesion	Epicarditis PRV IHC staining in myocyte and leukocyte	Epicarditis and myocarditis. PRV IHC staining in myocyte and leukocyte	Epicarditis and myocarditis.
IFN 1 and downstream (Mx Viperin) response	No response	Moderate increase	High increase	High/moderate increase	High/moderate increase
Antibody development **µ1c and µNS-specific IgM**	Non detected	Non detected	Significant increase of µNS ab	Significant increase of µNS and µ1c ab	Significant increase of µNS and µ1c ab

EIBS Erythrocytic inclusion body syndrome is a viral disease of Chinook and Coho salmon. The disease has been reported in the USA and Japan [57, 96]. The clinical findings of the disease consist of an elevated number of inclusions in red blood cells which precede the establishment of anemia in the affected fish. The severity of anemia span between moderate to severe; the peak of the anemia in the individual fish correlates with the peak of viral infection in the blood [57]. The major histopathological findings of EIBS-affected Coho salmon are necrosis of the muscle fibers in the cardiac ventricle and atrium [135]. Under field condition, EIBS outbreak is followed by increased severe mortality. Virions were observed in EM in the intracytoplasmic inclusions of erythrocyte of fish affected with EIBS, and a viral etiology was suggested [136], and later confirmed by experimental infection with purified PRV-2 particles [57].

PRV-3 disease was described in 2013 as a new disease in Rainbow trout in Norway [45]. The outbreak occurred in the freshwater flow-through farm in fish of approximately 200 gr. Affected fish showed loss of appetite and lethargy, which was followed by mortality. The skin and gills were pale, skin hemorrhages and bilateral exophthalmos was often observed. Necropsy findings revealed a general circulatory failure, with pale heart and sometimes dilated, blood-filled atrium. The peritoneal cavity contained sero-hemorrhagic ascites. The liver was regularly pale or yellowish and the kidney and spleen were swollen. The histopathological investigation revealed that the organs mainly affected were the heart, red skeletal musculature, and liver. Heart histopathology of affected fish revealed pancarditis (endo-, myocarditis, and epicarditis): in most severe cases necrotic lesions were also observed. In a few cases, necrotic lesions replaced by fibrosis were observed in red muscular fibers. The liver of affected fish showed focal to multifocal vacuolization and necrosis of hepatocytes [45]. The sequence of a PRV related virus was discovered in that outbreak, and later PRV-3 has been characterized, the experimental trial demonstrated to be the agent of this disease [56, 63].

The disease has been initially named “HSMI-like” [45] however there are clear differences with the HSMI, and PRV-3 disease is the name currently proposed.

In 2017, disease outbreaks with severe mortality in RAS in Denmark were associated with PRV-3. Affected fish showed neurological symptoms with uncoordinated swimming behavior and loss of balance. Clinically affected fish suffered severe anemia. Histopathological changes in the heart were partly overlapping with those of the previously described disease outbreak in Norway. In affected farms, PRV-3 was not the only pathogen detected, and other infections might be present such as (IPNV, *Flavobacterium psychrophilum,* and *Renibacterium salmoninarum*). In average, on a farm direct mortality rate is around 20%; however, in severe cases, the whole RAS unit have been emptied.

Under experimental condition it has been possible to induce, histopathological lesions in Rainbow trout, consistent with the first field outbreak (Fig. 15) using infected blood [63] or purified viral particles (Vendramin et al., in press). A parallel experiment has also been conducted in Brown trout, being suspected as natural host reservoir of the infection (Vendramin et al. under preparation). PRV-3 infection in Rainbow trout follows the kinetics of an acute infection characterized by a peak of the virus 3-4 weeks after exposure followed by clearance from the host. The innate immune

response expressed through the IFN response is elicited once the virus reaches its peak in the target cells, the erythrocytes. By applying the bead-based immunoassay developed for PRV-1 in Atlantic salmon, it has been possible to detect specific antibodies to PRV μ1C in Rainbow trout challenged with PRV-3, starting from 2 weeks after the peak of viremia.

PRV-3 disease possibly has different clinical manifestations; the field outbreak occurred in Norway and the output of the experimental challenge, depict PRV-3 as an acute infection in Rainbow trout which enhance the development of pancarditis in the later phase of infection. Under field condition in RAS in Denmark, environmental conditions such as water quality and the co-presence of other infections such as *F. psychrophilum*, *R. salmominarum,* and IPNV, drives the host-pathogen interaction into an acute form where anemia is a dominant finding.

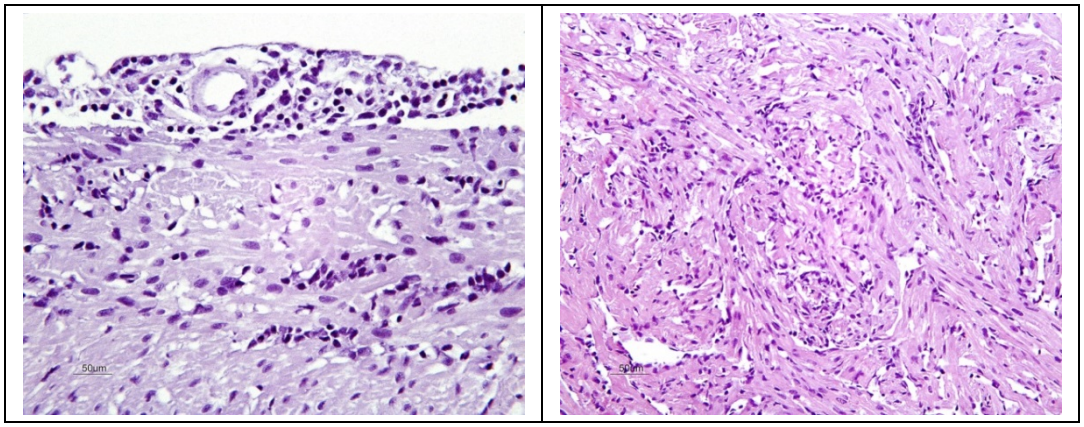

Figure 15. Heart tissue of Rainbow trout infected with PRV-3. Epicarditis (on the left) and myocarditis in compact and spongious layer (on the right) Pictures from Anne Berit Olsen [63]

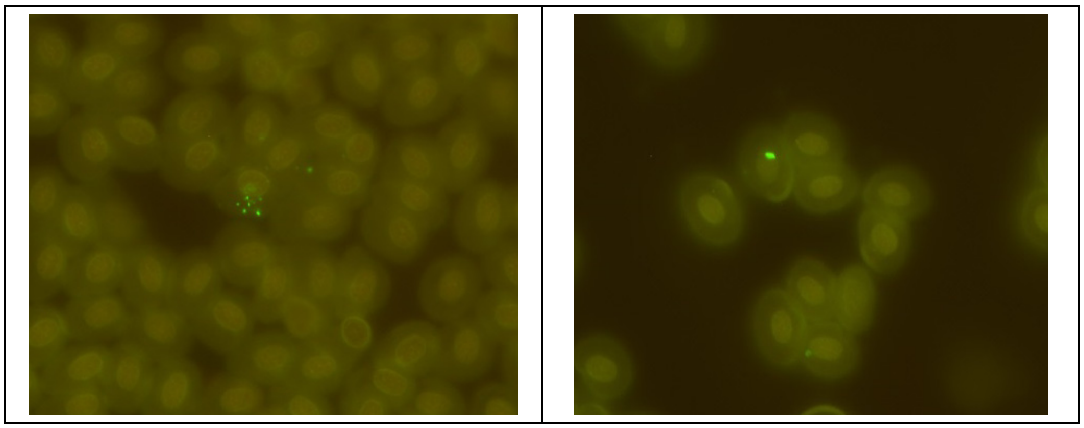

Figure 16. Staining of PRV-σ1 antigen in intracytoplasmic inclusions of RBC of PRV-3 infected Rainbow trout.[63]

Other PRV associated diseases/conditions.

Jaundice syndrome occurs in several salmonid species including Atlantic salmon, Chinook salmon, Coho salmon, and Rainbow trout. No obvious aetiological agent has been isolated so far. The disease has in the past been associated with toxicity to antibiotic treatment against BKD [137], to exposure to industrial effluents [138], and finally, Coho salmon with Jaundice were diagnosed with EIBS, and therefore a viral infection suggested as aetiology [139]. Jaundice syndrome is defined as the acute systemic disease; its most prominent clinical sign is the skin discoloration of affected fish which show yellow staining of the abdominal region. Affected fish suffer mortality after 1 to 2 days from the appearance of clinical signs. The disease is reported to occur sporadically in salmon farming in British Columbia and cumulative mortality associated with this syndrome, albeit low (<1.5% per production cycle), can significantly affect a single pen [140]. Histopathological findings include necrosis in liver, kidney and endocardial hypertrophy [140]. The aetiology of Jaundice syndrome in salmonid is still debated. In one experimental trial, PRV was tested as a potential agent [140]; IP challenged fish were sampled 155 days post infection. None of the fish displayed histopathology consistent with Jaundice syndrome while testing PRV positive. These results lead to ruling out PRV as the aetiological agent. However it is known that PRV infects red blood cells [99], and it could be speculated that Jaundice syndrome observed in Chinook salmon infected with PRV-1 and Coho infected with PRV-3 [66, 101] are downstream consequences of the clearance process of infected erythrocytes. Notably, a recent study localizes PRV-1 by in situ hybridization to heart, liver, and kidney of Chinook salmon diagnosed with Jaundice syndrome [75].

Discoloration of fish fillet. PRV-1 has been associated with the development of melanized spot in Atlantic salmon fillet [76]

Diagnostic tools for PRV-mediated diseases

Three different types of diagnostic tools are currently available to investigate PRV infection.

Molecular tools**.**

qPCR for viral RNA detection. For all three PRV subtypes, qPCR protocols for the detection of the L1 segment are available [45, 57, 64]. These molecular tools are suitable for viral screening and surveillance programs. Each protocol is specific for each PRV-subtype. Being PRV infecting and replicating in red blood cells, target organs can be blood or highly perfused organs such as heart and spleen [63].

In situ hybridization for PRV-1 has been recently established [64, 75], this tool is useful in the localization of the pathogen in archive material (paraffin embedded tissue). A specific PRV-3 probe has been developed at the end of this project and tested on samples from experimental challenge conducted in Rainbow trout.

Histopathology and Immunohistochemistry.

These techniques are valuable in clinical disease outbreaks. The histopathological criteria to assess heart in HSMI outbreak and PRV-3 disease are well defined [94] (Vendramin et al. 2018 submitted). Polyclonal antibodies have been successfully used for the staining of PRV-1 antigens in formalin fixed tissues [141]. For EIBS blood smear observation and hematocrit assessment might provide valuable tools during clinical outbreaks.

PRV Antibody detection.

Bead-based immunoassay is available for the detection of PRV-1 antibodies in Atlantic salmon [134], this test has also been successfully tested for the detection of PRV antibodies in Rainbow trout infected with PRV-3 (Vendramin et al. 2018 submitted). Upon validation, this might be a valid tool for the screening of a large population in farmed stocks.

PRV and immune response

Innate immunity

The innate immune system in salmonids plays a significant role in antiviral protection. It is the first line of defense against viruses, rapid and can limit viral replication until an adaptive response can be mounted. It is particularly important in coldblooded animals for which the kinetics of immune response is highly dependent on environmental parameters. Similar components and pathways of the innate immune system to those of higher vertebrates have been found in salmonids [142]. For orthoreoviruses, the intracellular exposure of the dsRNA genome represents an important pathogen-associated molecular pattern (PAMP) to activate the innate immune system [143].

Double-stranded RNA viruses induce IFN type 1 response [143, 144]. This immunological pathway is central in the protection against viral infections in vertebrates including fish [145, 146].

PRV-1 infection in Atlantic salmon [126] and PRV-3 in Rainbow trout [63] (Vendramin et al.,2018 submitted) can induce IFN type 1 pathway and IFN inducible genes, such as Mx and Viperin, as measured by immune gene expression. Notably, the induction of the IFN response is detected around the time PRV infection peaks in red blood cells.

After the core particle has entered the cytosol and replication starts, the PRV replication soon is organized in viral cytoplasmic structures called viral factories. They start out as numerous, small granulae, which gradually increase in size but decrease in numbers. If the viral replication is not interrupted, the dsRNA will not be exposed. The dsRNA of the incoming core particle is not revealed as the primary transcription of viral mRNA occurs within the core particle. The ejected mRNA is identical to the positive strand of the dsRNA genome, and it becomes encapsidated before synthesis of the negative strand occurs. This happens within the viral factories, where the necessary viral building bricks gather. The viral dsRNA is therefore not necessarily available for dsRNA recognizing Toll-like receptors (TLR3 and TLR22) present in the endosome [147] or for the cytoplasmatic receptors (RIG-I and MDA5) [148]. However, cytopathic effects, autophagy, misfolded viral proteins, lack of successful encapsidation and trapping of dysfunctional erythrocytes by splenic macrophages are examples that can produce an exposure of the viral dsRNA.

In the splenic filter, infected erythrocyte can be trapped and lyzed leading to release of dsRNA molecule in the extracellular space. Free dsRNA can bind to Class-A scavenger receptors (SR-As). These receptors have been described in mammals and fish, are able to bind dsRNA and internalize them [149], delivering the dsRNA molecules to intracellular sensors [150, 151].

Once dsRNA is internalized endoplasmic and cytoplasmic PRRs are able to recognize the molecule.

The viral mRNA, although capped, is not polyadenylated, and thus different from cellular cytosolic RNA. The effect of the latter in trigging innate response is unknown.

Remarkably the IFN cascades triggered by PRV are described with different magnitude. The experiment conducted with PRV-1 originated from Norwegian HSMI outbreak induced a strong immune response in Atlantic salmon [39, 126, 131, 152, 153] whereas experiments conducted with

strains from British Columbia induce minimal or negligible IFN response in Atlantic and Sockeye salmon [133, 140, 154]. However, the timing of the samplings in the latter experiments may have influenced the results. PRV-3 induce IFN pathway in Rainbow trout to a comparable magnitude than PRV-1 (HSMI) in Atlantic salmon [63].

Notably, the activation of the IFN cascade is not limited to measurement of immune gene expression; it has been shown that IFN mediated immune response, induced by PRV infection is able to modulate the susceptibility of Atlantic salmon to heterologous viruses such as SAV and IHNV [131, 152].

In fish virology, another remarkable example of this pathway is represented by IPNV which can modulate the susceptibility of Rainbow trout to subsequent challenges with rhabdovirus [155, 156].

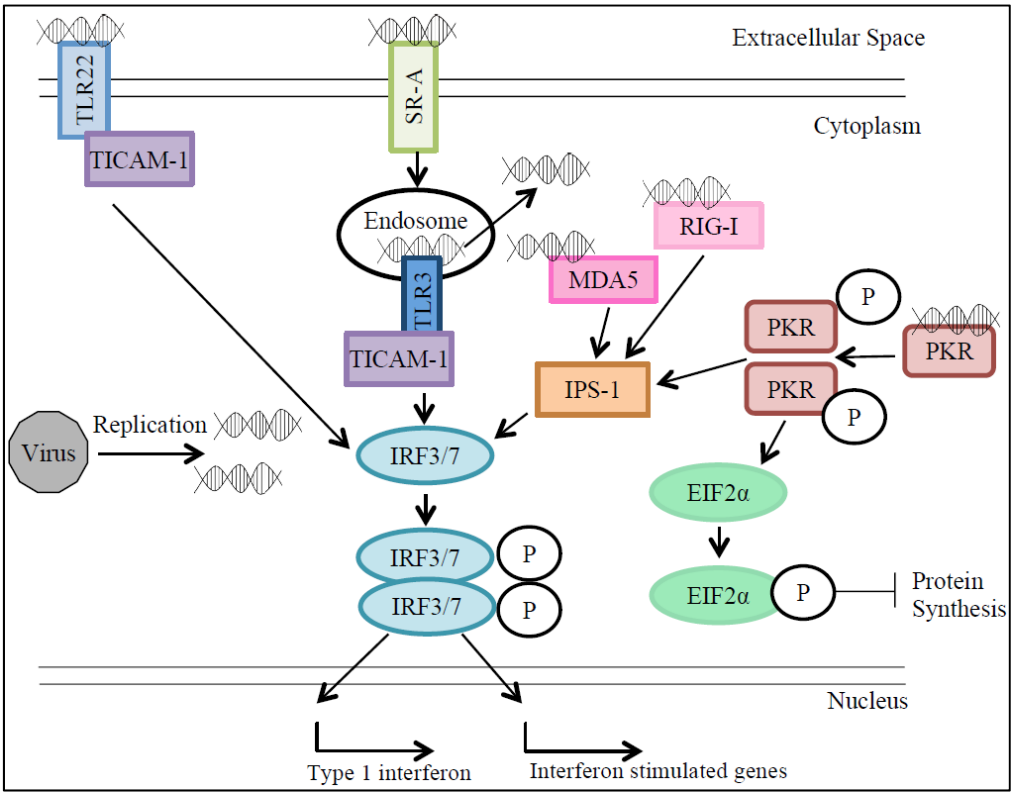

Figure 17. Viral dsRNA initiate antiviral pathways. Picture modified from Pointer 2014 [157]

Adaptive immunity

The adaptive antiviral immune response is characterized by an antigen-specific response which enables immunological memory. The immunological memory is pivotal for the specific and effective response in tackling a new infection, and this concept is widely used in the development of vaccines and management practices for disease prevention.

Two distinct cellular components are involved in the adaptive response; IgM+ and IgT+ B cells are responsible for the production of the humoral – specific antibody production, and CD8+ T-cells are pivotal for the cytotoxic cell-mediated immune response.

In Atlantic salmon and Rainbow trout, specific antibody production in the context of viral infections is widely documented [158–160]. It has also been shown that combining serological methods and viral detection increase the sensitivity of the surveillance program for viral diseases [161]. By *in-vitro* expression and purification of recombinant proteins of PRV [55, 99, 134, 141], it has been possible to establish an immunological assay to detect PRV specific antibodies in Atlantic salmon plasma [134]. Under experimental conditions, PRV infected fish develop specific antibody response for the outer capsid μ1 and the non-structural μNS protein 6 weeks after challenge. Antibody response is detectable at least until 10 weeks after challenge. Despite the development of a specific immune response, PRV-1 establish as a persistent infection. Similarly to PRV-1, another naked, dsRNA virus, IPNV establish a persistent infection in Atlantic salmon and Rainbow trout [162, 163].

T-cell cytotoxic immunity response after PRV-1 infection in Atlantic salmon is correlated with the development of heart inflammatory changes. In the experimental trial, it has been observed that in Atlantic salmon challenged with PRV, subsequently to viral peak (occurred 7 wpc), there was a marked increase in CD8+ T cell infiltration and myocarditis (10 wpc) developing in the experimentally challenged fish [132].

In Rainbow trout, cell-mediated cytotoxicity (CMC) has been observed during viral infections with *Viral haemorragic septicaemia virus* [164]. In the cell-mediated response to VHSV, MHC class I cells were involved, and an increase transcriptional level of CD8α and Natural Killer cells enhancement factor were measured supporting the presence of functional cytotoxic CD8+ lymphocytes.

Duration of viral infectivity outside the host

Physical-chemical factors confer resistance of the viral infectivity outside the host. The lack of susceptible cell lines has hampered the study of viral infectivity studies of PRV. The resistance of the virus to different treatment relies on the purification of virus from infected blood cells and *in vivo* trial to check infectivity and viability after treatment.

Naked virus particles are generally more resistant outside the host than an enveloped virus. An aquatic fish virus, which could be possibly used as a guideline for viral infectivity studies, is IPNV. Furthermore, a comparison to physicochemical properties of warm-blooded orthoreovirus can provide important insights.

An orthoreovirus isolated from turkey was found to be very stable virus, not susceptible to chloroform, nor to IUdR (10 M 5-iodo-2'-deoxyuridine) treatment, acid pH (3.0), finally partial reduction of one Turkey isolate (2 log) was observed after heat treatment (56 degrees for 30 minutes) [165].

IPNV is also a resistant virus in the environment [166]. Heat treatment (over 40 °C) inhibit replication, while heating infected material at 65°C for 1 minute inactivate more than 99.99% of the viable particles. The virus is very resistant to low pH, whereas 10 minutes of treatment at high pH (12) can inactivate more than 99.9% of the virus. In distilled water, hypochlorite at a concentration of 0.1 mg Cl /1 inactivated IPNV within 60 seconds [167].

Disinfectants compounds which contain active substances such as halogens, oxygen-releasing compounds, and acids and alkalines, are all able to inactivate at least 99.99 % of IPNV in five minutes. On the contrary quaternary ammonium compounds had no virucidal effect on IPNV. Iodophors, broadly disinfection of salmonids eggs, are less sensitive than chlorine compounds to the presence of organic material; 100 mg/1 free iodine inactivated a 99.9-99.99% of IPNV in 10 min when disinfecting eyed eggs of salmonids. Formalin (0,2% for 1 h), malachite green (5mg/l for 60 min), thimerosal (0,2% for 10 min) have negligible effect [166].

AIMS OF THE STUDY

The main aim of this study was to gain knowledge on the PRV subtypes present in Denmark with emphasis on subtype 3 (PRV-3). Once the project began, PRV subtypes classification was not defined yet. Soon after the discovery of a new disease in Rainbow trout, PRV- 3 genome has been described and compared to PRV-1, leading to the current classification. This is the reason why the project is divided into two chapters, the main one focusing on PRV-3 and the secondary one, on PRV-1.

Subgoals

1. **Pathogenesis study of PRV-3 infection causing heart pathology in Rainbow trout and Atlantic salmon.**

2. **Genetic and antigenic characterization of PRV-3 from Rainbow trout.**

3. **Experimental trials to demonstrate the causative relationship between PRV-3 infection and heart pathology in Rainbow trout.**

4. **Map PRV-1 in wild salmonid populations in North Atlantic Ocean.**

5. **Investigate interference in Atlantic salmon in co-challenge with PRV-1 and IHNV.**

SUMMARY OF PAPERS

Paper 1

Infection experiments with novel *Piscine orthoreovirus* from Rainbow trout (*Oncorhynchus mykiss*) in salmonids

Helena Hauge*, Niccoló Vendramin*, Torunn Taksdal, Anne Berit Olsen, Øystein Wessel, Susie Sommer Mikkelsen, Anna Luiza Farias Alencar, Niels Jørgen Olesen, Maria Krudtaa Dahle

*These authors contributed equally to this work

PLoS ONE 12(7): e0180293 2017

This study assess the risk associated with the introduction of the PRV-3 to farmed Rainbow trout and Atlantic salmon by studying infection and disease pathogenesis, aiming to provide more diagnostic knowledge. In experimental trials, intraperitoneally injected PRV-3 was shown to replicate in blood in both salmonid species, but more effectively in Rainbow trout. In Rainbow trout, the virus levels peaked in blood and heart of cohabitants 6 weeks post challenge, along with increased expression of antiviral genes (Mx and Viperin) in the spleen, with 80–100% of the cohabitants infected. Heart inflammation was diagnosed in all cohabitants examined 8 weeks post challenge. In contrast, less than 50% of the Atlantic salmon cohabitants were infected between 8 and 16 weeks post challenge and the antiviral response in these fish was very low. From 12 weeks post challenge and onwards, mild focal myocarditis was demonstrated in a few virus-positive salmon. In conclusion, PRV-3 infects both salmonid species, but faster transmission, more notable antiviral response and more prominent heart pathology were observed in Rainbow trout.

Paper 2

Molecular and Antigenic Characterization of *Piscine orthoreovirus* (PRV) from Rainbow Trout (*Oncorhynchus mykiss*)

Dhamotharan Kannimuthu, Niccoló Vendramin, Turhan Markussen, Øystein Wessel, Argelia Cuenca, Ingvild Bergman Nyman, Anne Berit Olsen, Tortsein Tengs, Maria Krudtaa Dahle, Espen Rimstad.

Viruses 2018, 10(4)

In this study, molecular and antigenic characterization of PRV-3 was performed. Virus particles were purified by gradient ultracentrifugation and the complete coding sequences of PRV-3 were obtained by Illumina sequencing. When compared to PRV-1, the nucleotide identity of the coding regions was 80.1%, and the amino acid identities of the predicted PRV-3 proteins varied from 96.7% (λ1) to 79.1% (σ3). Phylogenetic analysis showed that PRV-3 belongs to a separate cluster. The region encoding σ3 were sequenced from PRV-3 isolates collected from Rainbow trout in Europe. These sequences clustered together but were distant from PRV-3 that was isolated from Rainbow trout in Norway. Bioinformatic analyses of PRV-3 proteins revealed that predicted secondary structures and functional domains were conserved between PRV-3 and PRV-1. Our findings indicate that despite different species preferences of the PRV subtypes, several genetic, antigenic, and structural properties are conserved between PRV-1 and PRV-3.

Paper 3

Piscine orthoreovirus subtype 3 (PRV-3) causes heart inflammation in Rainbow trout (*Onchorynchus mykiss*)

Niccoló Vendramin*, Dhamotharan Kannimuthu*, Anne Berit Olsen, Argelia Cuenca, Lena Hammerlund Teige, Øystein Wessel, Tine Moesgaard Iburg, Maria Krudtaa Dahle, Espen Rimstad, Niels Jørgen Olesen

*These authors contributed equally to this work

In press at Veterinary Research 2019

In this study, we conducted a 10-week long experimental infection trial in Rainbow trout with purified PRV-3 particles and demonstrated the causal relationship between the virus and the development of heart inflammation. Monitoring the PRV-3 load in heart and spleen by RT-qPCR showed a progressive increase of viral RNA to a peak, followed by clearance. The development of characteristic cardiac histopathological findings occurred in the late phase of the trial and was associated with increased expression of CD8+, indicating cytotoxic T cell proliferation.

The findings indicate that, under these experimental conditions, PRV-3 infection in Rainbow trout act similar to PRV-1 infection in Atlantic salmon with regards to immunological responses and development of heart pathology, but not in the ability to establish a persistent infection.

Paper 4

Presence and genetic variability of Piscine orthoreovirus genotype 1 (PRV-1) in wild salmonids in Northern Europe and Atlantic Ocean

Niccoló Vendramin, Argelia Cuenca, Juliane Sørensen, Anna Luiza Farias Alencar, Debes Christiansen, Jan Arge Jacobsen, Charlotte Axen, François Lieffrig , Neil M. Ruane, Patrick Martin, Timothy Sheehan, Tine Moesgaard Iburg, Espen Rimstad, Niels Jørgen Olesen

Piscine orthoreovirus genotype 1 (PRV-1) is widespread in farmed Atlantic salmon (*Salmo salar* L.) populations in Northern Europe, Canada and Chile. PRV-1 has been detected in wild fish in Norway and Canada, however little information of its geographical distribution in wild populations is currently available and the effect of PRV-1 infection in wild populations is currently unknown. In this study we present the findings of a survey conducted on 1076 wild salmonids sampled in Denmark, Sweden, Ireland, Faroe Islands, France, Belgium and Greenland between 2010 and 2017. PRV-1 is reported for the first time in Denmark, Sweden, Faroe Island and Ireland. The PRV-1 prevalence varies from 0% in France, Belgium and Greenland to 50% in Faroe Islands. In total 66 positive samples tested positive for PRV-1, including Atlantic salmon broodfish returning to spawn and Atlantic salmon collected at the feeding ground north of Faroe Islands. The phylogenetic analysis of S1 sequences of the PRV-1 isolates obtained in this survey show lack of philogeography. This study help shedding light on the spread and genetic diversity of the virus identified in populations of free living fish and provide rationale for screening wild broodfish used in re-stocking programs.

Paper 5

Piscine orthoreovirus infection in Atlantic salmon (_Salmo salar_) protects against subsequent challenge with infectious hematopoietic necrosis virus (IHNV).

Niccoló Vendramin, Anna Luisa Farias Alencar, Tine Moesgaard Iburg, Maria Krudtaa Dahle, Øystein Wessel, Anne Berit Olsen, Espen Rimstad, Niels Jørgen Olesen

Vet Res. 2018 ;49(1):30.

The aim of this study was to investigate the interactions between a primary PRV-1 infection and a secondary IHNV infection under experimental conditions. A PRV-1 cohabitation challenge was performed with Atlantic salmon. At the peak of PRV-1 viremia the fish were challenged by immersion with an IHNV genogroup E isolate. Clinical signs and morbidity were monitored. Target organs were sampled at selected time points to assess viral loads of both pathogens. Antiviral immune response and presence of histopathological findings were also investigated. Whereas the PRV-negative/IHNV positive group suffered a significant decrease in survival caused by IHNV, the PRV infected groups did not suffer any morbidity and showed negligible levels of IHNV infection. Antiviral response genes were induced, as measured in spleen samples, from PRV infected fish prior to IHNV challenge. In conclusion, PRV-1 infection protects Atlantic salmon against IHNV infection and morbidity, most likely by inducing a protective innate antiviral response.

METHODOLOGY

The following section includes considerations regarding material and methods used in the present study with a focus on the experimental *in vivo* challenge and next-generation sequencing. Information regarding methods as Real-Time RT-PCR, histopathology, IFAT are referred the enclosed papers.

Experimental *in vivo* challenge

The capacity of establishing and refining challenge model *in vivo* to study host-pathogen interaction of non-cultivable pathogen has been a significant asset of this project.

Facility. All experimental challenges have been performed in the close contained tank facility of DTU-Aqua in Lyngby (formerly located at DTU-VET in Frederiksberg- Copenhagen). The experimental facility includes 3 separate units; a closed unit based on full recirculation where naïve fish are produced periodically; a quarantine unit, and a highly contained facility where it is possible to conduct experiments with exotic and highly infectious pathogens. All water effluent from the contained facility is heat treated (120°C for 2 minutes) before released into the municipality system. In the experiments (paper 1, 2, 3, 5) fish have been maintained in 180lts transparent tanks (Fig. 18).

Figure 18. Experimental tanks used in all experimental trials

All tanks are covered with a lid to avoid fish to escape and spill of water from one tank to another. The tank volume is divided into two parts by a plate located at the bottom of the cylinder. This allows biological particles to gather in the tip of the cone, increasing water quality and cleaning efficiency while reducing water renewal. The day-light ration was 12:12 and artificially provided. Water and air flow can be adjusted for individual tanks.

Fish source. Eyed-eggs from a commercial fish farm with high health status (VHS, IHN, BKD, IPN free) are introduced in the facility, disinfected and hatched. Good management practice such as proper feeding rate, maintenance of good water quality and hygiene practices are implemented. These procedures have allowed the introduction of pathogen-free fish in the experiments.

Inoculum. It is currently impossible practically to measure the amount of infective PRV in the inoculum. Therefore, the level of PRV RNA is estimated by Ct value. When using infected tissue, this might be biased by the presence of both viral genomic RNA and messenger RNA. Another critical point is to minimize the risk that the infected material used in the inoculum is harboring other unwanted pathogens.

For PRV-3, the inoculum has been produced *in vivo* by IP-injecting SPF fish with blood collected from the disease outbreak in the field. Fish from this outbreak were screened for PRV-1, infectious salmon anaemia virus (ISAV), salmonid alphavirus (SAV), piscine myocarditis virus (PMCV), viral haemorrhagic septicaemia virus (VHSV), infectious hematopoietic necrosis virus (IHNV) and infectious pancreatic necrosis virus (IPNV) with negative results. The material produced for paper 2 and 3 has been then screened by NGS, demonstrating only the presence of PRV-3 in the infected blood and Cs-Cl gradient purified viral particles.

For PRV-1 the inoculum used in paper 5 has been provided by Lund and colleagues [152] and thoroughly screened.

The material has been collected early at the peak of PRV RNA level in blood. In the initial phase of replication in blood, the amount of infective virus and viral RNA (PRV Ct value) correlate; in the late phase of infection, this correlation is lost after the peak of infection in blood cells, probably due to suppression of viral protein translation [58].

The preparation of the inoculum and quantification method of PRV RNA is described in detail in the papers (Paper 2, 3 and 5).

Challenge model. Three different challenge models have been used along the Ph.D. project, intraperitoneal injection (IP), cohabitation, bath challenge.

1. Bath challenge has been used for IHNV challenge in Atlantic salmon in paper 5. The experimental model for this fish rhabdovirus is well established, and the availability of susceptible cell culture allows to control how many infective viral particles are added to the water [168].
2. IP challenge has been used to produce PRV-3 infected blood in the experiment of paper 1, 2 and 3. The purpose of these experiments was to allow effective replication of PRV-3 in naïve Rainbow trout to produce blood with high virus load virus purification and further challenge. By IP injection mucosal immunity is bypassed enhancing fast and efficient pathogen replication; furthermore this method allows standardization of the dose
3. Cohabitation model has been widely used in studying PRV-1 infection in Atlantic salmon [39, 152]. The model provides a suitable model for studying horizontal transmission and by mimicking natural infection and is thus suitable for pathogenesis studies. This model is particularly suitable with a highly infective and transmissible pathogen like PRV, therefore it has been utilized for experiment in paper 1, 3, 5.

Consideration of 3R's

Before conducting trials on experimental animals, all researchers need to consider how the concept of the three R's can be implemented in the experimental design according to Russel and Bruch (1959) [169]. According to the National Center for the 3 R's [170], these criteria stand for replacement, reduction, and refinement.

- Replacement: replace sentient animals with alternative methods such as lower vertebrates or *in vitro* system. The lack of suitable cell culture for PRV has driven to the "*in vivo*" work the focus of the project.

- Reduction: refers to any strategy that allows in diminishing the number of animals used to obtain sufficient data to answer the research question. Alternatively, the concept of reduction is meant as a strategy that allows increasing the information obtained per animal and per animal. Thus reduction is a concept that allows limiting or avoiding the subsequent use of additional animals, without compromising animal welfare. In order to assess the virulence of emerging disease such as the case of PRV-3 infection in salmonids, we have included a number of fish sufficient to conduct sufficient sampling even if reduced survival of 50% was occurring. This prudent approach was formulated on the following rationale. If the challenge trial reduces the survival rate for more than 50%, it is a highly pathogenic virus. If reduced survival is between 0 and 50% it is a priority to obtain data on viral pathogenesis.

- Refinement: optimizing the welfare of the experimental animals throughout the study. The aim is to minimize the number of experimental animals used in research and to optimize the welfare of the experimental animals. As described in material and methods of paper 2, 4 and 6, the husbandry practices, such as animal density, water renewal, feeding ratio have fulfilled the requirement to guarantee animal welfare. Daily surveillance during the experimental time has minimized the risk of onset of mortality, and protocols were established to euthanize fish showing clear clinical signs (the occurrence which did not occur). Finally prior to each blood sampling fish were anesthetized, in case of tissue sampling fish were euthanized by an overdose of anesthetics.

Next-generation sequencing NGS

Next-generation sequencing platform is based on immobilization of DNA/RNA onto a solid support, cyclic sequencing reaction through automated fluidic devices, and detection of molecular events by imaging [171].

In this project, NGS has been used to obtain the full genome of newly discovered PRV-3 (paper 2). Plasma from infected Rainbow trout has demonstrated to be an excellent starting material. NGS is a technique that is independent of the target sequence. But in order to obtain good coverage of the virus, it is important to analyze a sample that is rich in the virus pathogen with a limited presence of the host genetic material. Plasma contains a small amount of host DNA and RNA due to break down of cells or pathogens. Furthermore in plasma collected at the peak of infection, measured as low Ct value by qPCR assumedly only virions contribute to the qPCR. Therefore this matrix is rich in particles with full genome packed in dsRNA which represent an optimal starting material for obtaining full genome sequences.

As mentioned, NGS is un-specific, meaning that depending on the depth of the read, all genetic material purified in the sample is amplified and sequenced. We have taken advantage of this feature to screen the efficiency of the purification process in paper 2, therefore, ensuring that only PRV-3 was inoculated in the shedder fish of paper 3.

In our study total RNA extracted from 2 mL of pooled plasma originating from two individuals (Ct 25.78 and 26). RNA was added 0.1 volumes of 3 M sodium acetate (pH 7.5) and 2X volumes of 100% ethanol and mixed gently. Macrogen (Seoul, Korea) performed library preparation using the TruSeq RNA Library Prep Kit v2 (Illumina Inc., San Diego, CA, USA), followed by whole genome de novo sequencing (101 bases, paired-end reads) on an Illumina HiSeq4000 platform [172] (1/7th lane). Sequences were de novo assembled using the genome assembler software SPAdes (version 3.10.1) [173].

Table 4. Number of reads targeting each PRV-3 segment from the Illumina HiSeq4000 run

Segment	L1	L2	L3	M1	M2	M3	S1	S2	S3	S4
No. of reads	24936	20588	21162	10256	10733	10461	4378	5961	5213	4034

RESULTS AND GENERAL DISCUSSION

This project started soon after the first disease outbreak caused by the virus that later was named PRV-3. Little was known about the virus, its epidemiology or pathogenesis of the infection. The project aimed to characterize the newly discovered virus. We purified the virus from fish blood, obtained full genome sequence, and characterized antigenic properties of virus proteins comparing them to homologous proteins of PRV-1. Furthermore, we demonstrated the causative relationship between the infection with PRV-3 in Rainbow trout and the development of heart pathologic lesions. The output of this project can be used to assess the risk posed by a putative emerging disease in Rainbow trout aquaculture.

In order to gain knowledge on PRV epidemiology in general, including the distribution of the PRV-1 subtype in Europe, we investigated the presence of PRV-1 in wild Atlantic salmon in Denmark and in the North Atlantic. This would add information about the prevalence of this pathogen outside salmon farming countries and on viral ecology. Due to the high prevalence of PRV in farmed Atlantic salmon, we finally assessed the potential interference that this widespread virus could cause on the listed disease IHN caused by a *rhabdovirus* considered as a potential threat for Atlantic salmon aquaculture, by challenging Atlantic salmon with PRV and then subsequently with IHNV.

PRV-3 is a new PRV-subtype

Upon description of a new disease in Rainbow trout associated with *Piscine orthoreovirus*, this project focused on the characterization of the newly discovered isolate.

In paper 2 we focused on providing genetic and antigenic characterization of the PRV variant from Rainbow trout. ICTV provides guidelines for classifying new species within genus *Orthoreovirus*: the nucleotide sequence identities of homologous genome segments should be <60% equal to other orthoreoviruses, the amino acid sequence identities for more conserved proteins <65% and for more divergent outer capsid proteins <35% [174].

Genome characterization. PRV-3 genome is closely related to PRV-1. We obtained 106,257,190 Illumina reads (53,128,595 pairs), and *de novo* assembly produced a large number of contigs from a template consisting of purified virus. There was a high degree of amino acid sequence similarity to PRV-1 for many contigs. PRV-3 genome consists of 10 gene segments, similar to the orthoreoviruses.

Table 5. General features of proteins encoded by genome segments of PRV-3 and percentage of identity of nucleotide and amino acid sequences between PRV-1, 2 and 3. Protein nomenclature is based on PRV-1 [55]

Segment	Protein name	Predicted Function	PRV-3 & PRV-1		PRV-3 & PRV-2		PRV-1 & PRV-2	
			nt	Aa	nt	aa	nt	aa
L1	λ3 (Core RdRp)	RNA-dependent RNA polymerase	80.9	95.2	76.1	88.4	77.3	89.0
L2	λ2 (Core turret)	Guanylyltransferase, methyltransferase	77.8	90.0	70.5	77.1	70.9	76.9
L3	λ1 (Core shell)	Helicase, NTPase, RNA triphosphatase	80.3	96.9	78.4	93.1	77.8	92.9
M1	μ2 (Core NTPase)	NTPase, RNA triphosphatase, RNA binding	78.4	88.7	71.7	78.3	72.2	78.1
M2	μ1 (Outer shell)	Outer capsid protein, membrane penetration	81.2	91.5	76.5	84.4	76.6	85.3
M3	μNS (NS-factory)	Non-structural protein	76.5	82.2	61.6	59.7	62.9	59.3
S1	σ3 (Outer clamp)	σ3: outer capsid protein, zinc metalloprotein	80.5	79.1	71.3	69.7	71.6	69.7
	p13	p13: cytotoxic, integral membrane protein	85.6	78.2	78.1	63.7	77.3	62.9
S2	σ2 (Core clamp)	Inner capsid, RNA binding protein	80.4	88.8	70.1	73.8	70.2	77.1
S3	σNS (NS-RNA)	Non-structural protein	87.9	94.6	77.8	85.3	76.6	84.7
S4	σ1 (Outer fibre)	Cell attachment protein	80.1	81.6	64.1	64.4	65.5	66.7

At nucleotide level, the similarity between PRV-3 and PRV-1 is >76%; while between PRV-3 and PRV-2 is >64%. At amino acid level, the similarity between PRV-3 and PRV-1 is >78%; while

between PRV-3 and PRV-2 is >64%. Therefore the species criteria are not fulfilled by the new PRV variant, which cannot be considered a new orthoreovirus species. The isolate is defined as new PRV-subtype together with PRV-1 and PRV-2. PRV-3 is the name proposed nomenclature.

Antigenic properties. PRV-3 proteins have been tested with antibodies raised against homologous PRV-1 proteins. A panel of anti PRV-1 proteins included: anti-σ1 [141], anti-σ3 [55], anti-σNS [58], anti-μ1C [141], anti-λ1 and anti-PRV-1 (antiserum against purified PRV-1 particles) [39]. Western blot analysis of PRV-3 infected blood cells, the samples were denatured in the process, demonstrated that polyclonal antisera of rabbit immunized with homologous proteins of PRV-1 cross-reacted with the PRV-3 proteins σ1, σ3, σNS, μ1, μNS, and λ1. Conversely, an attempt to stain in IHC the PRV-3 antigen failed (paper 1 and 3). This could be explained by the low sensitivity of the antibodies in IHC, i.e., the amount of viral antigen was too low for detection. Furthermore, it is possible that conformational differences between PRV-1 and PRV-3 proteins do not allow antibody binding. The PRV-3 antigen can be detected in western blots under denaturing conditions, but it highlights the need for the development of specific tools for identifying the localization of PRV-3 in tissue. PRV-3 infection in Rainbow trout, elicit production of PRV specific antibodies against μ1C (paper 3) which can be detected by a bead-based multiplex immunoassay developed for PRV-1 [134].

The different PRV subtypes prefer different host species.

PRV-1 is ubiquitous in farmed Atlantic salmon in Norway during the sea phase [58, 65]. The virus is also reported in farmed Atlantic salmon in Faroe Island, Iceland, Scotland, Ireland, and Canada (British Columbia) and in the Atlantic and Coho salmon farmed in Chile [66]. The first detection of PRV-1 in farmed Atlantic salmon in Canada dates back to 1987, and from a retrospective study, there are suspected detection of PRV-1 in Steelhead in 1977 [88]. Recently, the virus has also been detected with moderate low prevalence in several wild salmonids belonging to *Onchorynchus* species such as Coho salmon, Chinook salmon, Pink salmon and Steelhead (Rainbow trout) [89].

HSMI was reported for the first time in 1999 [94]. The detection of the virus in samples origination prior to this date implies that the pathogen and its host have co-existed before the appearance of the disease. What has triggered the emergence of the disease? This question is still unanswered, but likely, a combination of changes in the farming practices including feed, and in virus and host properties has led to this development [8]. Notably, despite farming Rainbow trout and Atlantic salmon in close proximity in net pens, no HSMI outbreak has ever been described in farmed Rainbow trout in Norway, suggesting the Rainbow trout susceptibility to PRV-1 is low.

In Norway, the virus has been detected with a prevalence of 10% in wild Atlantic salmon [67, 86]. However, it is not known if the wild salmon caught the infection from the farming activity or fellow wild fish. The phylogenetic analysis of PRV-1 subtype isolates has shown a certain genetic variability [66, 83] and reassortment between PRV-1 strains in the wild has been described [67]. On the background of the sequence variability of segment S1, 4 genotypes within PRV-1 has been provided [67]. However, this classification needs to consider that the viral genome is segmented, and genotyping on one segment might not reflect the correct phylogenetic position of one isolate once reassortment occurs.

The causative relationship between PRV-1 infection and HSMI development has been questioned due to the detection of PRV-1 in the wild and farmed population not showing HSMI [88, 133]. One possible explanation for this controversy could refer to variation in virus virulence between strains, i.e., in the ability to cause HSMI after PRV-1 infection. Notably, it has been shown that PRV-1 isolates detected in HSMI outbreaks (with one exception) forms a cluster genetically distinct from PRV-1 detected in Canada [39, 75].

In paper 4 we investigated the prevalence of PRV-1 in wild stock of Atlantic salmon and sea migrating brown trout (*Salmo trutta*) in several European countries facing the Atlantic Ocean and Western Greenland. PRV-1 was detected by qPCR only in Atlantic salmon and S1 segment sequenced from Denmark, Sweden, Ireland, and the Faroe Islands. The virus has not been detected, although investigated, in wild salmonid fish in France, Belgium and Greenland, although with lower prevalence compared to the assessment conducted in Norway.

Notably, we report the first detection of PRV-1 in southern Sweden (Mörrum Å). Being Atlantic salmon from the Baltic Sea, which has been segregated from other Atlantic salmon stocks, this finding poses an epidemiological question. However, Mörrum Å is located in the southern part of the Baltic Sea, possibly making the transfer through Kattegat a possibility; no re-stocking activities are reported to occur in this river. Possibly marine fish, prey of Atlantic salmon, might increase the ability of the virus to spread in different epidemiological areas [90]. Predation of PRV infected escaped Atlantic salmon from wild fish such as Cod (*Gadus morhua*) has been speculated as the source of transmission from farmed to wild fish [91].

In two Danish rivers that host the Atlantic salmon population, Ribe Å located on the west coast, and Guden Å on the east coast, a mixed PRV-1 population has been detected. In these rivers, the S1 sequences clustered both with Norwegian isolates (of HSMI outbreaks) and with Canadian isolates (not related to HSMI). These findings describe conditions which could allow viral reassortment to occur in wild populations [67].

Interestingly, the prevalence of PRV-1 in wild fish in Denmark has decreased over the years. The estimated prevalence at the beginning of the screening period in 2013 was approximately 10%, however, in the screening conducted in 2017, no PRV-1 was detected in the wild Atlantic salmon returning to the river and used as broodstock. Although several factors can affect this trend, it has to be underlined that improved management procedure is implemented in the re-stocking units, accurate egg-disinfection minimize the risk of egg-associated transmission in the progeny of the broodstock and PRV-free juveniles are released in the rivers.

So far, PRV-2 and EIBS have not been reported in Europe, and experimental studies conducted earlier have shown that the susceptibility of Rainbow trout is relatively low [97]. This could indicate that the risk of introduction of this pathogen in Rainbow trout aquaculture is low.

The detection of PRV-3 prompted us to search for the origin and the natural reservoir of PRV-3. One hypothesis, which requires further investigation, is that PRV-3 has co-evolved with brown trout as PRV-1 has in Atlantic salmon. Notably, neither PRV-3 viruses nor their associated diseases were reported in Pacific salmon species, nor in the Pacific where Rainbow trout originate from. Recently, however, the Jaundice syndrome in Chinook has been linked to PRV-1 infection [75] and similar for PRV-3 and Coho [66]. The disease outbreak caused by PRV-3 could generate the output

of an infection of a new host after the introduction of Rainbow trout in Europe, which occurred in 1879 [175]. Some findings support this theory, i.e., the detection of PRV-3 in asymptomatic brown trout (*Salmo trutta*) in Italy and a high prevalence of PRV-3 in wild brown trout used for re-stocking in Denmark. The susceptibility of brown trout to PRV-3 has been further demonstrated under experimental conditions (Vendramin et al., in preparation). Finally, PRV-3 might be associated with brown trout suffering Proliferative darkening disease (PDD), in alpine rivers in Switzerland and Germany [176]. These findings warrant further investigation in order to shed light on the evolution of the virus and its host range.

In this project (paper 1), we have assessed the susceptibility of Atlantic salmon to PRV-3 infection. In this host, replication of PRV-3 in injected fish occurred with low efficiency. Instead of the peak phase followed by a significant decrease of the virus load observed in Rainbow trout, PRV-3 load did not increase in Atlantic salmon and from week 4 started to decrease. In Atlantic salmon cohabitants, the infection rate was never higher than 50% of fish sampled per time point. Only occasional fish showed mild heart pathology in the Atlantic salmon experiment, and no IFN gene induction was observed. These findings support the hypothesis of a lower susceptibility of Atlantic salmon to PRV-3.

Each of the currently known three viral subtypes of PRV preferably interacts with one fish species. The diseases share the main target cell, the erythrocyte; whereas pathologic lesions develop predominantly in three organs/cells, erythrocyte (PRV-2 and PRV-3 disease), heart (PRV-1 and PRV-3) and muscle (PRV-1). The description of cell receptors, which allow the virus to enter the host cells, will provide a functional explanation of the interaction.

Table 6. Overview of PRV subtypes interaction and fish hosts

	Atlantic salmon	Brown trout	Rainbow trout	Pacific Salmon (Coho-Chinook-Sockeye)
PRV-1	**HSMI**	N/A	N/A	Jaundice (Chinook)
PRV-2	N/A	N/A	N/A	**EIBS (Coho)**
PRV-3	Experimental	**PRV-3 Disease**	**PRV-3 disease**	Jaundice (Coho)

Surveillance and diagnostic procedures for PRV-3

In cohabitation challenge trials in Rainbow trout and Atlantic salmon, we scrutinized a panel of tissue from infected fish and selected target organs for conducting surveillance and diagnostic.

Tissue samples of heart, head kidney, spleen, and blood were collected along an 8 weeks trial and tested. The highest viral RNA load was observed in blood and spleen in the early phase of infection.

In the experiments conducted in Rainbow trout (paper 1, 2, 3) we have shown that PRV-3 is detected at low Ct value in blood and highly perfused organs such as spleen and heart. Furthermore, we have stained PRV σ1 in blood smears from highly infected Rainbow trout cohabitants (paper 1); the antigen was localized in inclusions in the cytoplasm of erythrocyte, resembling viral factories as described for PRV-1 [59]. Finally, we detected PRV-3 antigen in western blot from blood pellet of cohabitants at the peak of infection (paper 3).

These findings show that erythrocyte is the cell type where the virus replicates, as described for PRV-1 [99] in Atlantic salmon, providing scientific-based evidence for establishing guidelines for diagnostic and surveillance procedures.

For surveillance purposes (detection of the virus in population), blood or highly perfused organs shall be tested by qPCR [45].

For disease diagnostic, it is necessary to associate the viral RNA detection to histopathological findings. With the current knowledge, the target organ to investigate is the heart and findings are overlapping with the ones described for HSMI, including (endo-, myocarditis, and epicarditis).

PRV-3 infection is acute in Rainbow trout and causes heart pathology

After i.p.-injection of PRV-3 in naïve Rainbow trout, the virus replicates and reaches an acute peak phase after 3–4 weeks (at 12°C). Horizontal transmission was shown in the co-habitation challenge where the naïve cohabitant fish were infected soon after the virus peak in the shedders, suggesting rapid horizontal transmission of the virus and framing the shedding phase during the peak of viraemia (Fig. 19). In paper 1 and 3 we show how PRV-3 causes an acute infection in Rainbow trout. In cohabitation challenge, virus peak 3-4 weeks post-injection in shedders; soon after the peak, the number of positive fish per time point decreased, consistently, the Ct values of the positive fish increased, suggesting clearance of the infection. In cohabitants, the kinetics of infection follows the same trend, delayed with two weeks compared to shedders.

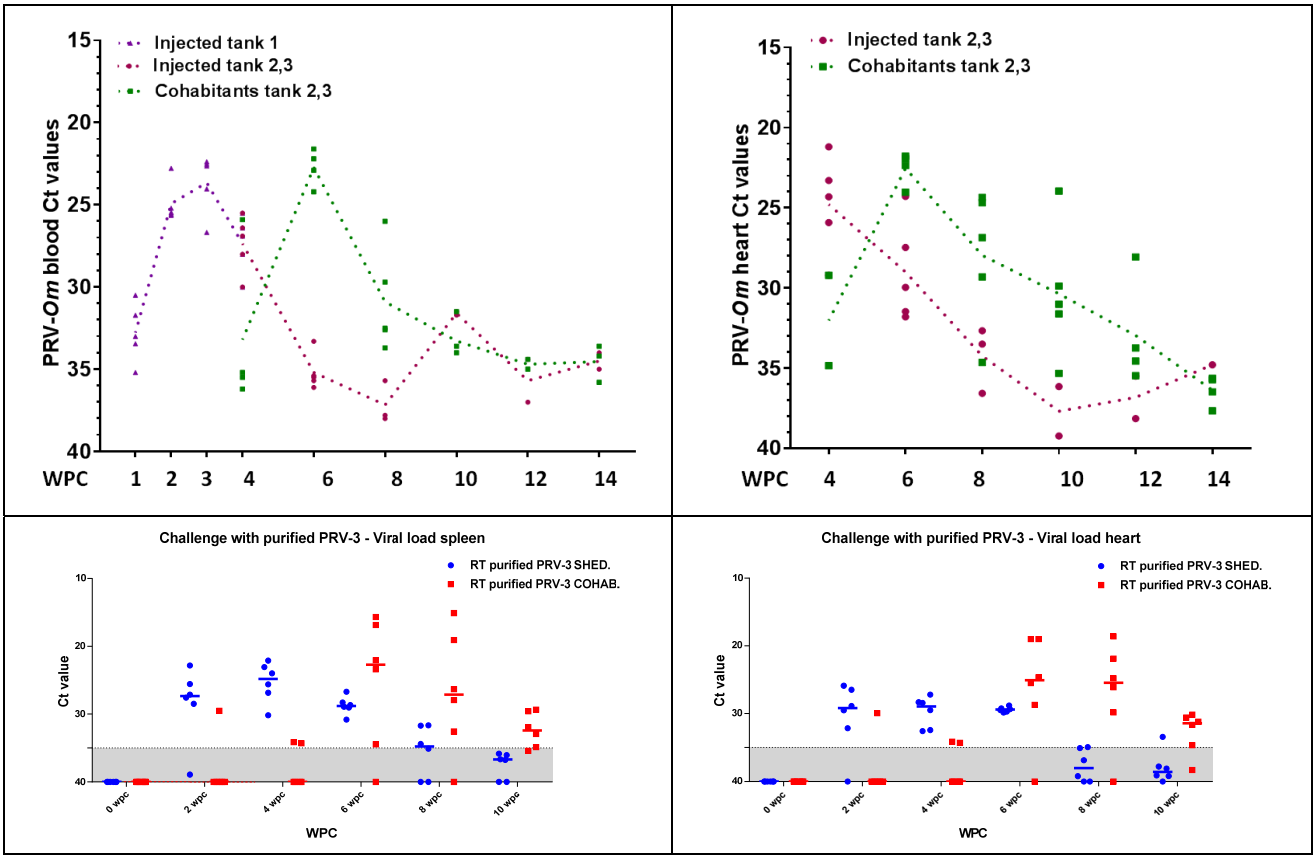

Figure 19. Top. PRV-3 RNA load in blood (left) and heart (right) during the cohabitation challenge experiment with PRV-3 infected blood (paper1). [63]
Lower row, PRV-3 RNA load in spleen (left) and heart (right) during cohabitation challenge experiment with purified PRV-3 particles (paper 3) (Vendramin et al. 2019 in press)

Conversely, PRV-1 establishes a persistent infection in Atlantic salmon as shown in Paper 5 and previously reported [39, 58, 133]. PRV-1 load reaches the maximum load, approximately 4 weeks post challenge in cohabitants. Interestingly, in both infections heart pathology develops after the viral peak, with a delay of 2 weeks after the viral peak [63, 141].

Currently, the most investigated PRV mediated disease is HSMI, the clinical manifestation of PRV-1 infection in Atlantic salmon. HSMI is a chronic disease of Atlantic salmon, mediated by the host inflammatory immune response after PRV-1 infection [39, 94].

EIBS is another disease mediated by PRV. This is an acute disease caused by PRV-2 in Coho salmon, anemia is the most prominent clinical finding, which shortly follows the peak of viremia [57, 139].

In the experimental studies conducted within this project, PRV-3 infection in Rainbow trout leads to the development of heart pathology resembling HSMI (paper 1 and 3) [45, 63]. Interestingly, in both experimental challenges conducted with PRV-3 (paper 1 and 3), viral replication appears to be more efficient in cohabitant fish compared to IP injected shedders. This finding is quite surprising; in IP injected fish mucosal barriers and related immunity are by-passed. Furthermore, in the experimental tank IP injected fish act as shedders but also as cohabitants compared to other shedders increasing the viral load towards they are exposed. A factor to consider is that, in shedders, what is delivered by injection is likely a mixture of virion, ISVP and core particles, whereas cohabitants are exposed to mature infective virions.

It can be speculated that the virus, by infecting the host through the natural route, has the opportunity to replicate in mucosal cells at the port of entry before erythrocytes are infected. IP injected the virus, on the other hand, faces the immune system at the peritoneal level, which may modulate the replication of the virus. It is described that peritoneal exudate cells have immune capacity such as opsonization against live *A. salmonicida in vitro* [177]. Furthermore, phagocytes, including granulocytes (particularly neutrophils) and mononuclear phagocytes (tissue macrophages and circulating monocytes) which are present in the peritoneal exudate, act as antigen presenting cells, and their stimulation improves efficacy resistance towards disease challenge [178]. In a recent experimental vaccination-challenge trial against SAV in Atlantic salmon, it was shown how an adjuvant based on CpG and Poly I:C can improve the performance of the vaccine [179]. Notably CpG oligonucleotides and Poly I:C can stimulate the production of pro-inflammatory cytokines/chemokines and Type I IFNs, which increase the host's ability to eliminate viral pathogens [180]. Type I IFNs is also stimulated by dsRNA. In IP injected fish, Rainbow trout are potentially exposed to PRV-3 dsRNA, which can activate IFN innate immune response (paper 3) of fish interacting with the ongoing infection and potentially interacting with the second wave of infection from virus internally or shed by other fish.

In paper 3, to prove a causative relationship between PRV-3 infection and development of heart pathologic lesions, Rainbow trout were exposed in cohabitation trial with purified PRV-3 particles. In another tank, PRV-3 infected blood was used as positive control.

Due to the lack of susceptible cell cultures, PRV-3 was propagated *in vivo* and purified on CsCl gradient following an established protocol for PRV-1 [39]. EM and NGS confirmed the presence of virus particles and the purity of the inoculum. This process is time- and manpower-consuming, and provided that the process was successful, it allowed using only one dose. The PRV-3 inoculum was tested by qPCR to assess the dose, which was estimated as rather low (32,67 Ct value). Nevertheless, the virus was able to replicate and develop the acute infection as previously observed. PRV-3 was able to induce heart pathology in the late phase of the experiment, after the viremia as

described in paper 1. The development of heart inflammation after the viral peak is a feature shared with PRV-1 in HSMI [39, 141].

It can be argued that the number of fish developing heart pathologic lesions was relatively low compared to the positive control group and the experiment conducted in paper 1. However, it has to be remarked that heart lesions were described in the challenged fish after PRV-3 load peaked in the target organs, and the risk of having observed heart lesions resembling HSMI by hazard is considered minimal. If lesions observed were not linked to the effect of the virus, then it has to be assumed that heart lesions would be randomly distributed in the population and observed according to normal distribution in the sampled population, which was not the case.

Possibly the inoculum dose affected this. A potential future experiment to investigate dose-response will clarify this.

PRV infection induces IFN pathway

In fish, erythrocytes are nucleated and may act as immune cells during infection [124]. During orthoreovirus infection and replication, dsRNA is produced, but not necessarily exposed to the cell cytoplasm. During viral replication, the core particle is the initial machine for mRNA production [123]. The assembly of the progeny virions starts around the positive ssRNA and occurs in viral factories, orchestrated in the perinuclear cytoplasm by μNS protein [59].

IFN suppression at the cellular level is a strategy used by many viruses to evade host immune response. IFN suppression is an established strategy by dsRNA virus during replication as shown for IPNV VP1 [181]. In this regards, MRV proteins μ2 and σ3 have IFN antagonist activity [182, 183]; the activity of the homologous PRV protein has been tested *in vitro* without showing the same functionality [55]. σ3, though, is a RNA binding protein and may indirectly reduce the innate response [55].

When considering the host-pathogen interaction at the higher level, i.e., virus and fish, it has to be considered that infected erythrocytes are processed in the splenic filter and thereby putatively exposing PRV and its dsRNA to immune competent cells.

dsRNA can stimulate the production of interferon and activate protein kinase R (PKR). Immunological studies based on microarray analyses and qPCR found that the PKR and the fish-specific PKZ, a molecule acting as PKR in fish [184, 185], were increased in PRV infected salmon erythrocytes [126]. A significant up-regulation of PKR was observed in salmon erythrocytes following PRV infection *ex vivo* [100]. PRV infection caused moderate down-regulation of multiple genes for proteins with potential effects on cell shape, motility, and metabolism [130]. Notably, the IFN cascade genes induction is measured in relation to the viral peak. In this timeframe, leaking of dsRNA from infected in erythrocyte is a likely event.

Experimental challenge conducted [99, 126, 130] and paper 5, the development of heart pathologic lesions is associated with induction of IFN response in relation to the early phase, and with CD8+ in the late phase of infection. It has been demonstrated under the experimental condition that the cells

infiltrating the heart tissue was mainly CD8+T cells and the virus replication peaked a few days prior to the inflammatory response, characterizing HSMI as a T cell myocarditis [132].

Interestingly, the infectious trial conducted in different salmonids (Atlantic salmon and Sockeye salmon) with the PRV-1 strain from Canada failed to induce a measurable IFN response [133, 154].

The IFN pathway is pivotal in protection against viral infections in vertebrates, including Atlantic salmon [146, 156]. The induction of IFN response in Atlantic salmon by PRV-1 is able to modulate the susceptibility of Atlantic salmon to superinfection with heterologous viral pathogens such as SAV and IHNV [131, 152]. Conversely, in Sockeye salmon challenged with PRV-1 which failed to induce IFN, no modulation of a subsequent challenge with IHNV was observed [154].

The latter is not the only example of PRV subtype failing to induce IFN, in experimental challenge performed in Atlantic salmon with PRV-3 (paper 1), no induction of this pathway was observed [63].

In paper 1 and 3 we show that also in Rainbow trout PRV-3 can induce IFN pathway and downstream genes such as Mx and Viperin. The host specificity of the different PRV subtypes might provide further insight into the capacity of inducing IFN of PRV.

Teleosts are ectothermic animals and as such not able to control their temperatures. It has been shown that fish can respond to infectious disease by developing a behavioral fever. This mechanism induces the fish to shift from optimal temperature moving to a warmer area with increased oxygen consumption and reduction of oxygen dissolved in the water. The increase of environmental temperature boost the immune response and possibly modulate replication capacity of the pathogen [186]. Induction of behavioral fever has been shown in Zebrafish exposed to dsRNA [187]. It is reported that HSMI outbreaks tend to appear more frequently late spring-early summer [188] and it is documented that HSMI affected fish in sea cage are close to the surface [66]; similarly, Rainbow trout during disease outbreak associated with PRV-3 disease swim in close proximity of the surface. Possibly fish are searching for the warmest temperature available in the confined environment to develop a behavioral fever.

Table 7. Resume of the pathogenesis of PRV-3 infection in Rainbow trout cohabitants under experimental conditions

PRV-3 infection in Rainbow trout (Cohabitants)					
Time / Components	**2 w.p.c.**	**4 w.p.c.**	**6 w.p.c.**	**8 w.p.c.**	**10 w.p.c.**
PRV RNA Kinetics		Detection of PRV RNA (L1 segment)	PRV RNA peak (L1 segment)	Clearance process	Clearance process
PRV Protein Kinetics	Non detected	Non detected	Detected	Non detected	Non detected
Histopathology	No lesion	No lesion	No lesion	Epicarditis and myocarditis.	Epicarditis and myocarditis.
CD4 marker in heart	No fold change	5 fold increase	No fold change	5 fold increase	Significant fold increase (15 times)
CD8 marker in heart	No fold change	No fold change	No fold change	No fold change	Significant fold increase (100 times)
IFN 1 and downstream (Mx Viperin) immune	No response	Moderate increase	High increase	No response	No response
Antibody development µ1c -specific	Non detected	Non detected	Non detected	Raise and µ1c ab	Significant raise of µ1c ab

PRV-3 cause emerging disease for Rainbow trout

In disease outbreak occurring in RAS in Denmark, the viral kinetics resembles what observed in under experimental condition with the increase, peak and clearance (Fig 20). Notably, under field conditions, clinical signs and mortality are subsequent to the peak of viral load. In this frame, the pathogenesis appears to be shifted towards an acute disease manifestation, where severe anemia impairs the homoeostasis of the fish, leading to mortality. The presence of concomitant infections, common in Rainbow trout production, such as *Renibacterium salmoninarum*, *Flavobacterium psychrophilum,* and IPNV can possibly alter the host immune response including the immune response in the heart.

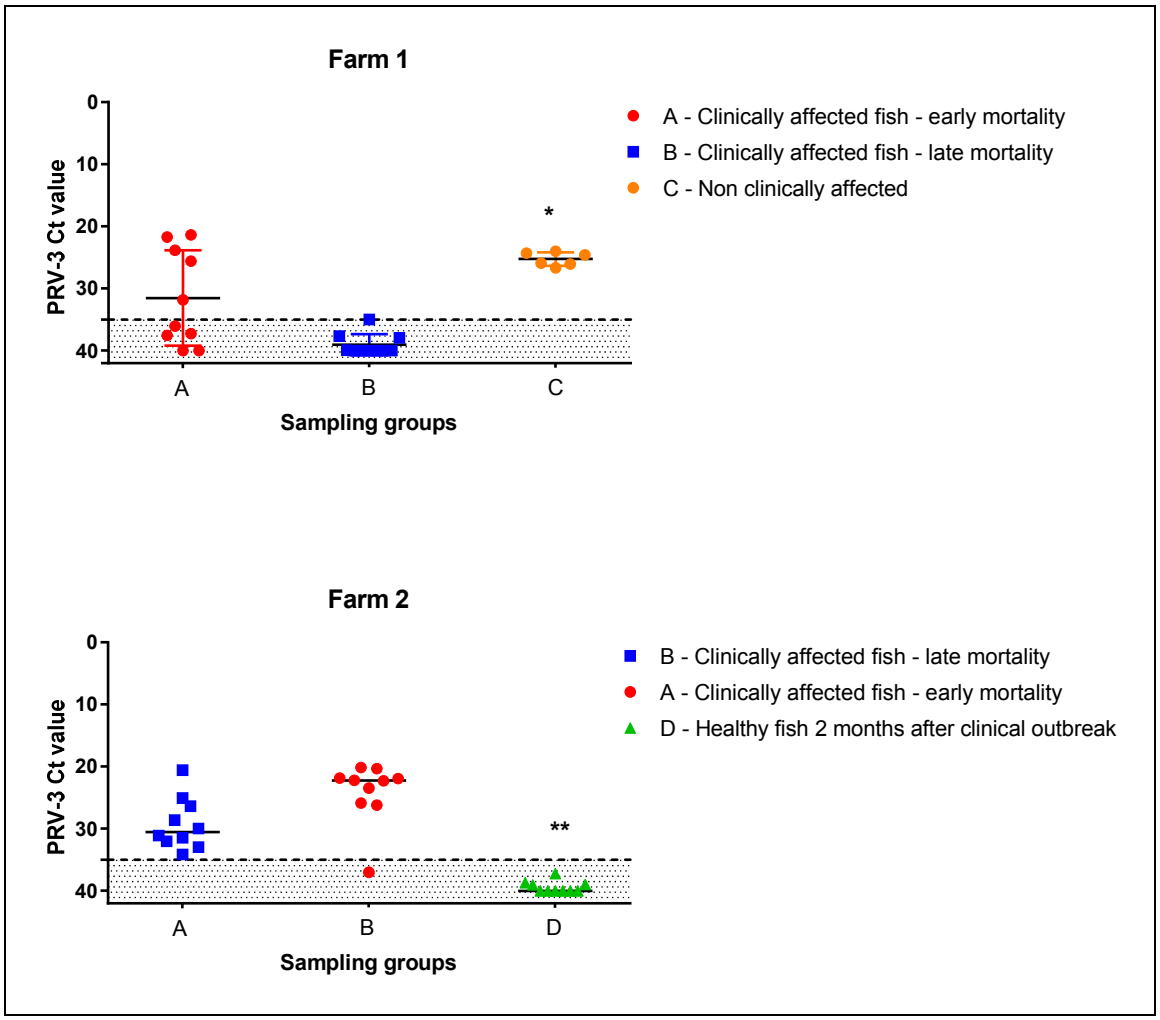

Figure 20. PRV-3 load in infected farms in Denmark.
In Farm 1, the group "Not clinically affected"* developed clinical disease related to PRV-3, 2 weeks after sampling. In farm 1 IPNV was isolated, and *Flavobacterium psychrophilum* detected in 3 out of 20 fish tested.
In Farm 2, healthy fish at sampling had suffered clinical outbreak 2 weeks prior to sampling, *Renibacterium salmoninarum* was detected in all 3 groups, whereas *Flavobacterium psychrophilum* was isolated from 9 out of the 30 fish tested. No other pathogenic bacteria were detected in Farms 1 or 2.

Table 8 and 9. Diagnostic reports in complex disease cases in Farm 1 and 2.
PRV-3 has been detected by qPCR; if median Ct is > 35 , value is displayed in the table as -/+ ; if median Ct value is 35<Ct<25 value displayed as ++; if median Ct is <25 value is displayed as +++.
Renibacterium salmoninarum is detected by ELISA.
IPNV isolated and identified in cell culture
Flavobacterium psychrophilum isolated on TYES agar.

FARM 1			
	Group A	Group B	Group C
PRV-3	++	-/+	+++
BKD	Neg	Neg	N/A
IPNV	+	+	N/A
F. psych	(+)	(+)	N/A

FARM 2			
	Group A	Group B	Group D
PRV-3	++	+++	-
BKD	Susp	Susp	Susp
IPNV	-	-	-
F. psych	+	+	-

The definition of emerging disease in aquaculture includes the appearance of a new pathogen, the occurrence of a known pathogen in a new species or the appearance of a disease in a geographical area where this was not previously reported [26]. PRVs are not cultivable *in vitro* and surveillance of fish viruses in Europe for the last decades has been conducted by cell culture; therefore the presence of the virus in native salmonid populations might have been overlooked.

In 2013, a new disease has been described in farmed Rainbow trout in freshwater in Norway, and PRV-3 firstly detected [45]. A number of disease outbreaks associated with this pathogen have been observed worldwide after this [56, 66, 87], and the virus has been found prevalent in Danish fish farms (Vendramin et al., in preparation). In this project, it has been possible under experimental conditions, to induce the heart pathology in Rainbow trout resembling that of the first field outbreak by using PRV-3 infected blood and PRV-3 purified particle (paper 1 and 3). The disease associated with PRV-3 has been firstly described in Norway in 2013 [45] and reported in 2017 in Scotland, Germany, and Denmark [56] therefore fulfilling the criteria for defining it as “emerging”.

CONCLUSIONS

In this project, we have gained significant knowledge of *Piscine orthoreovirus* infections in salmonids.

With specific reference to PRV-3, by establishing a reproducible challenge model under the experimental condition in Rainbow trout, it has been possible to investigate the pathogenesis of this virus, establishing guidelines for surveillance and diagnostic procedures.

The genome of the virus has been sequenced by NGS. The virus has been purified from infected erythrocytes and its antigenic properties tested by Western blot.

The diagnostic capacities and the awareness of this new virus have allowed implementing surveillance in Europe and effectively compiling the available knowledge on PRV-3 prevalence. These activities have provided the epidemiological picture of the distribution of the virus in Europe.

The challenge model, refined by testing the purified viral particles, has allowed demonstrating the causative relationship of the infection with heart pathology. This has provided scientific evidence for the classification of PRV-3 as emerging pathogen, responsible for disease outbreak in Rainbow trout farmed in recirculating aquaculture systems in Denmark.

We have further investigated the presence of PRV-1 in wild Atlantic salmon population in the Atlantic Ocean, describing the distribution of different PRV-1 clades in wild Atlantic salmon population returning to their home rivers. The documentation of a decrease in viral prevalence over time highlights the importance of implementation of disinfection procedures for eggs in the re-stocking operation of wild fish.

Finally, we have investigated viral interference in fish, showing how PRV-1 infection can protect Atlantic salmon from infection with IHNV, thereby characterizing the immune response to PRV-1.

In conclusion, when addressing an emerging disease, we have provided a case definition; we have demonstrated the causative agent and described the pathogenesis in susceptible hosts. Furthermore, we have contributed to validating the diagnostic tools. All these are fundamental steps to establish future control measures.

FUTURE PERSPECTIVES

The aquaculture production in RAS is deemed to increase in the future. The system is going to be exploited for high-value species such as Pike perch, Sturgeon for caviar production, Atlantic salmon, large Rainbow trout and shrimps. In Denmark, an increase in production in RAS system is expected to comply with environmental regulations; by 2026 all freshwater farms which produce more than 100 tons of fish per year, will have to implement recirculation (to different degrees) to limit their environmental impact [189].

In Norway, where sea lice and its treatment are currently limiting the development of aquaculture there is an increasing interest in RAS system; however, the price for energy needed to run the facility is limiting further development [190].

Overall, the aquaculture industry is following a trend where large corporate companies, which integrate the whole production chain, are replacing traditional, small-scale, family business.

This strategy has and will allow implementing diagnostic laboratories directly on site, which will overview the health status of the farm cluster. In this regard, the technological development occurred in the last decade with molecular biology allows nowadays to have portable qPCR devices, as well as sequencer of the size of a smartphone. The capability of real-time screening for a microbial population in farm and potential pathogens appear to be something that can be implemented in the near future.

This technology will need to be accompanied with in-depth knowledge on the "biological meaning" or the role that ubiquitous, opportunistic pathogens, such as PRV, have on the farmed population.

PRV-3 is currently posing serious challenges to Rainbow trout farming in RAS. In order to address them properly, acquiring knowledge on epidemiology and pathogenesis are essential to formulating preventive and control measures, these tasks are currently hampered by the lack of *in vitro* cultivation methods.

To achieve significant progress, it is needed to:

1) Develop diagnostic tools, which allow localizing the pathogen in the tissue of infected fish. These could be ISH probes or Mab, and will allow increasing resolution when dealing with complex diagnostic cases, understanding the role of the different pathogens insisting on the farm.
2) Assess the efficiency of egg-disinfection procedures. Being able to effectively disinfect eggs surface from PRV-free broodstock facility will allow the introduction of a pathogen-free juvenile in the farm.
3) Study Rainbow trout immune response to PRV-3 infection in different environmental conditions (water temperature) will possibly allow in RAS to modulate the exposure of the fish to the infection in a favorable condition for the host.
4) Understanding whether Rainbow trout mounts a protective immunity after exposure or not, will allow development of preventive prophylactic measures such as vaccines and controlled exposure to the pathogen.

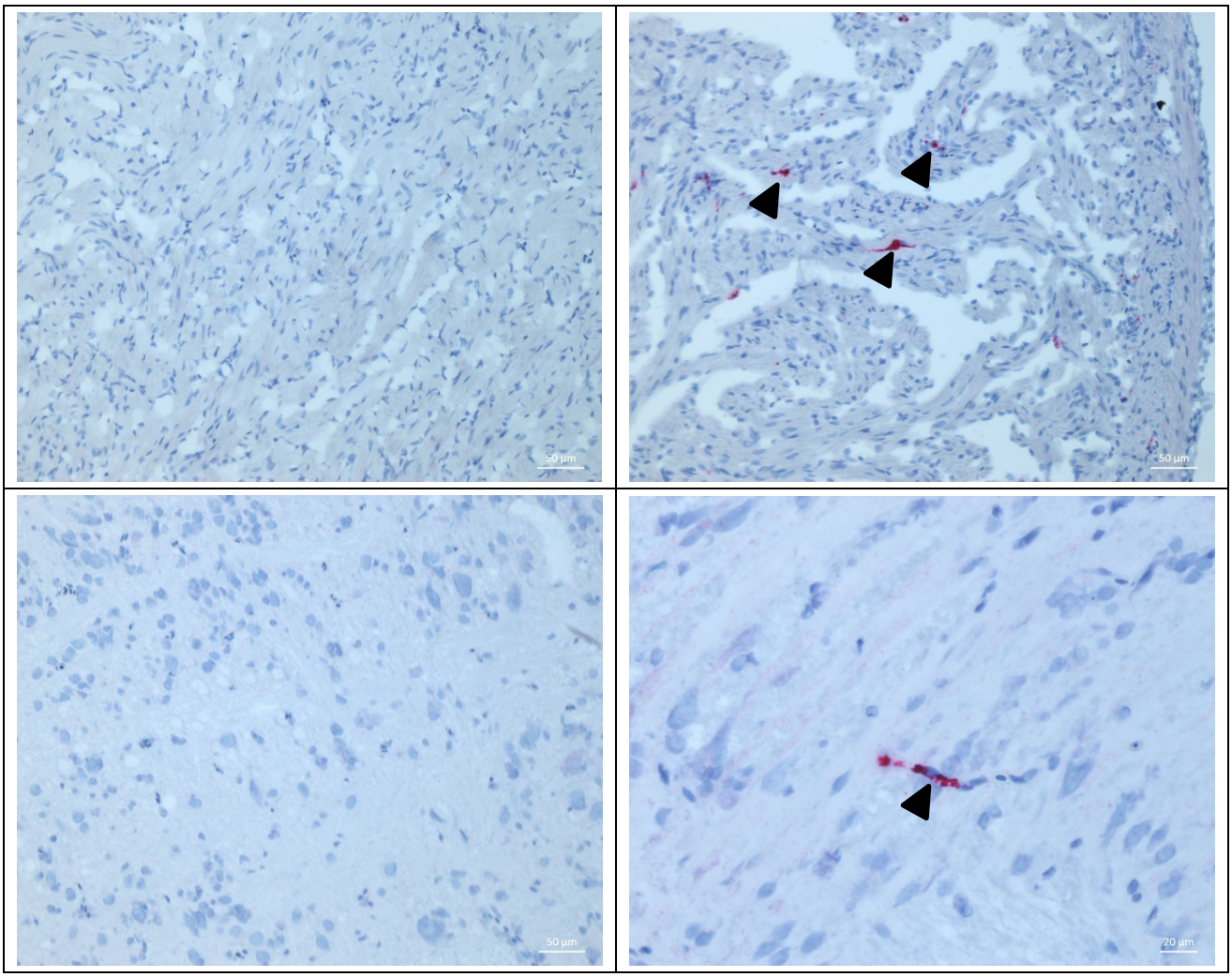

Figure 21. PRV-3 *In situ* hybridization staining (arrows) in cardiomyocytes (up right) and erythrocyte in capillaries in the brain (low right) of infected Rainbow trout. On the left, tissue from non-infected fish. Probe targeting L1 segment of PRV-3 developed with RNAscope®.

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Papers

Due to copyright, the original papers are not included in this version of the thesis. Please find references to and summaries of the papers on page 51-53.

In this project, we have gained significant knowledge of *Piscine orthoreovirus* infections in salmonids. A reproducible challenge model has been established to investigate the pathogenesis of PRV-3 infection in Rainbow trout and demonstrate the causative relationship of the infection with the development of heart pathology.

The genome of the virus has been fully sequenced by NGS. The diagnostic capacities and the awareness of this new virus have allowed implementing PRV-3 targeted surveillance providing the epidemiological picture of the distribution of the virus in Europe.

This has provided scientific evidence for the classification of PRV-3 as emerging pathogen, responsible for disease outbreak in Rainbow trout farmed in recirculating aquaculture systems in Denmark.

The presence of PRV-1 in wild Atlantic salmon population in the Atlantic Ocean has been further investigated, describing the distribution of different PRV-1 clades in wild Atlantic salmon population returning to their home rivers. The documentation of a decrease in viral prevalence over time highlights the importance of implementation of disinfection procedures for eggs in the re-stocking operation of wild fish.

Finally, we have investigated viral interference in fish, showing how PRV-1 infection can protect Atlantic salmon from infection with IHNV, thereby characterizing the immune response to PRV-1.

Technical
University of
Denmark

DTU Aqua
Kemitorvet
DK-2800 Kgs. Lyngby

www.aqua.dtu.dk